University of Alberta

The Characterization, Analyses and Biodegradation of Naphthenic acids

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfillment of the requirements for the degree of Master of Science

in

Microbiology and Biotechnology

Department of Biological Sciences

Edmonton, Alberta

Spring 2004

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ISBN: 0-612-96460-4
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ABSTRACT

"Naphthenic acids" comprise a group made up of hundreds of chemically and structurally related alkanoic compounds, which are natural components of bitumen. They are believed to be the main cause of acute toxicity of bitumen processing wastewaters, but also have commercial applications. The naphthenic acids distributions of extracts from several Athabasca oil sands ores, tailings ponds, as well as commercial mixtures were analyzed by gas chromatography-mass spectrometry of their *t*-butyldimethylsilyl derivatives. Statistical methods for comparing the resulting three-dimensional fingerprints were developed. An HPLC method was adapted for analysis of naphthenic acids concentrations in laboratory cultures. The biodegradation of Kodak salts and Merichem acids was characterized by measuring the rate of mineralization, naphthenic acids loss, toxicity decrease, and changes in component distribution.

ACKNOWLEDGEMENT

This project was made possible through funding from Syncrude Canada Ltd., Suncor Energy Inc., True North Energy, Albian Sands Energy, Canadian Natural Resources Limited, and the Canadian Water Network. The Natural Sciences and Engineering Research Council of Canada provided funding to purchase the HPLC.

I appreciate the previous work by Fervone Holowenko, who evaluated the GC-MS method; and Mark Chandra, whose Excel program allowed rapid analysis of large amounts of GC-MS data. I am indebted to: Tin-Wing Yen who made key contributions towards developing the HPLC method; Don Morgan, Dr. Randy Whittal, and especially Larry Harrow from the Chemistry mass spectrometry services who were willing to share their insight, support, and time; the group at Syncrude Canada Research Center for providing samples, support, and analysis; Dr. D.W. Smith and Maria Demeter for access to the Microtox™ instrument; Dr. N.G.N. Prasad for his help with the statistical analysis; Dr. Mingsheng Ma, who was a resource for the HPLC instrument. I would also like to express my appreciation to: Ruth Eckford, Rozlyn McInnis, and Leah Kirkpatrick, for the discussions. I also appreciate the support from people in the Microbiology and Cell Biotechnology research interest group including: Debbi Coy, Melissa Haveroen, Beth Hearn, Tom Hantos, Kathy Semple, Sara Ebert, Kathlyn Kirkwood, and Dr. Quanji Wu.

I am grateful for the time and energy invested by my committee members, Dr. Julia Foght, Dr. Selma Guigard, and Dr. Mike MacKinnon; as well as for their helpful discussions and suggestions. I am grateful to my supervisor, Dr. Phillip Fedorak, for this learning opportunity, as well as for his guidance, suggestions, encouragement, and patience.

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LIST OF ABBREVIATIONS

CI	Confidence interval
EDC	1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide
EI	Electron impact ionization, used in mass spectrometry
FT-IR	Fourier-transform infrared spectroscopy
GC-AED	Gas chromatography coupled to an atomic emission detector
GC-FID	Gas chromatography coupled to a flame ionization detector
GC-MS	Gas chromatography with mass spectrometry
HPLC	High performance liquid chromatography
IC_{20}	Concentration which decreases light ouput by 20% in Microtox ™ assay
IC_{50}	Concentration which decreases light output by 50% in Microtox™ assay
IR	Infrared spectroscopy
K_{ow}	Octanol-water partition coefficient
LC_{50}	Concentration lethal to 50% of the population
MLSBF	Naphthenic acid extracts from Mildred Lake Settling Basin
MS	Mass spectrometry
MTBSTFA	*N*-Methyl-*N*-(*t*-butyldimethylsilyl)trifluoroacetamide
NPH	2-Nitrophenylhydrazine
TAN	Total acid number
TIC	Total ion current
UV	Ultraviolet detection

1. INTRODUCTION

1.1 General Overview

1.1.1 Definition of naphthenic acids, physicochemical properties

Naphthenic acids are cyclic or acyclic alkanoic acids with the general formula $C_nH_{2n+Z}O_2$, where n is the carbon number and Z is a negative number, which represents the number of hydrogens lost from the saturated naphthenic acids (here "saturated" excludes the carbonyl group) as it becomes more unsaturated. The absolute value of Z divided by 2 gives the unsaturation number (excluding the carbonyl group); which is assumed to be the number of rings in the compounds. The rings may be fused or bridged. The acyclic components are highly branched, unlike fatty acids (Rudzinski et al. 2002), although fatty acids fit the formula $C_nH_{2n+Z}O_2$, for Z=0. Figure 1.1 shows examples of naphthenic acids structures. Besides the carboxylic acid group, cyclic naphthenic acids are also substituted with an alkyl group (R in Figure 1.1). The smallest cyclic structure would be a substituted cyclopentane, whereas the largest structure is expected to be less than 1000 Da (CONRAD 1998).

Mixtures of naphthenic acids are made up of hundreds of chemically and structurally related compounds. This, along with the complexity of the mixture makes separation, quantification, and identification of individual compounds extremely difficult. They are non-volatile, chemically stable, and act as surfactants. Salts of naphthenic acids are soluble in water. Naphthenic acids have dissociation constants that range between 10^{-5} and 10^{-6} (Brient et al. 1995), which is typical of most carboxylic acids (acetic acid = $10^{-4.7}$, propionic acid = $10^{-4.9}$, palmitic acid 10^{-8}) (Kanicky et al. 2000).

1.1.2 Crude oils contain naphthenic acids

Naphthenic acids are natural components of crude oils (Seifert and Teeter 1969, Seifert et al. 1969, Fan 1991, Tomczyk et al. 2001). They are present at different concentrations depending on the source of oil. Table 1.1 presents the percent by

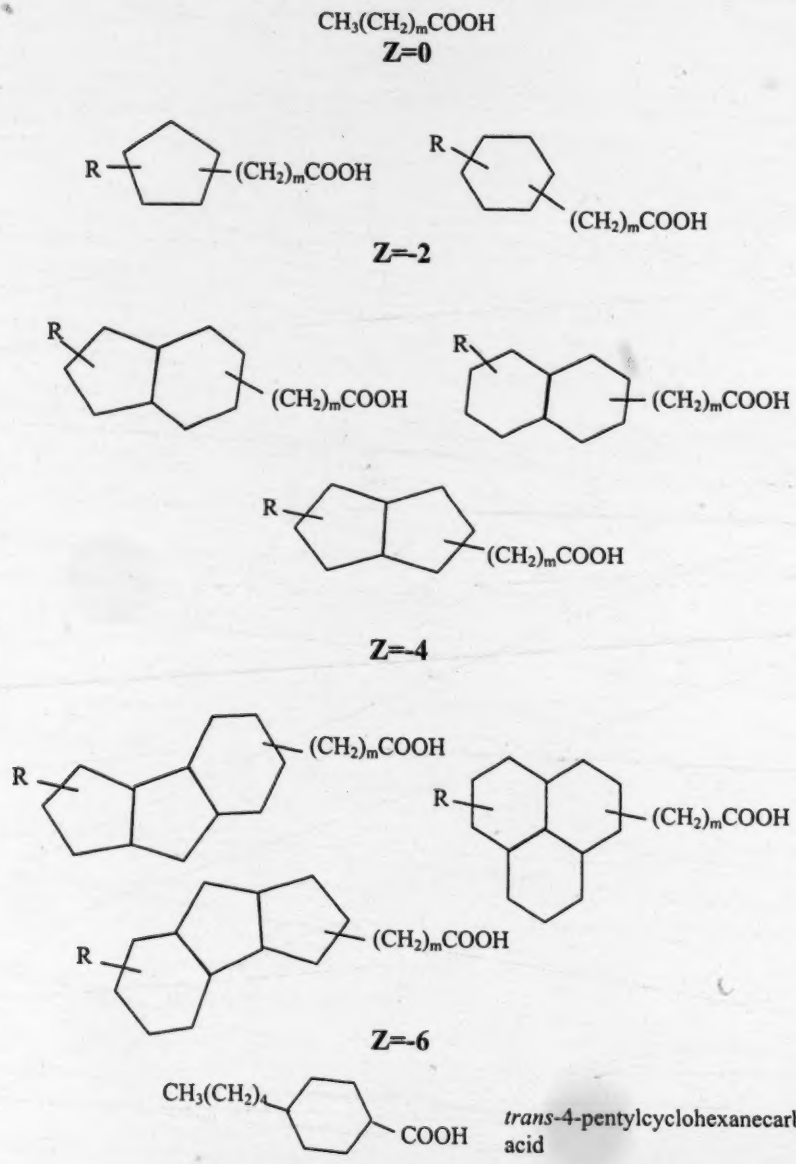


Figure 1.1 Sample naphthenic acid structures where R is an alkyl chain, Z describes the hydrogen deficiency, n is the carbon number, and m is the number of CH_2 units (Holowenko et al. 2001). The model naphthenic acid *trans*-4-pentylcyclohexane carboxylic acid has a carbon number =12 and Z=-2.

weight of naphthenic acids in different types of oil, where North American oils have the highest naphthenic acids concentration at 2% to 4%, and Egyptian crude has the lowest at 0.0015% to 0.403%. Carboxylic acids, which include naphthenic acids, are found in

petroleum because either the deposit has not undergone sufficient catagenesis or it has been biodegraded by bacteria (Tissot and Welte 1978).

Diagenesis and catagenesis result in petroleum formation (Tissot and Welte 1978). Diagenesis is mainly an anaerobic microbial process which involves methane production and condensation of biomolecules to kerogen (Tissot and Welte 1978). Catagenesis is a physical process involving high pressure and temperatures between 50 °C and 150 °C. Carbonyl groups, such as those found in carboxylic acids, are generally eliminated at this time and there is an increase in carbon to oxygen ratios (Tissot and Welte 1978). A deposit which has not undergone extensive thermal alteration would therefore contain a greater percentage of carboxylic acids. Exposure of petroleum to bacteria through processes such as earthquakes and erosion leads to its biodegradation (Riva 1983), which in turn leads to carboxylic acid formation. Carboxylic acids have been found in deposits of biodegraded oil (Nascimento et al. 1999, Meredith et al. 2000) and biodegradation of crude oil in laboratory experiments (Roques et al. 1994, Watson et al. 2002). Naphthenic acids in the Athabasca oil sands are produced by biodegradation of mature petroleum (Tissot and Welte 1978).

Naphthenic acids and other components in petroleum have structures similar to their biochemical precursors. For example, some bacteria produce lactobacillic acid, a C_9 fatty acid with a cyclopropyl subunit; and tuberculostearic acid, a methyl branched C_{19} fatty acid (White 2000). Methanogenic bacteria produce branched, ether-linked lipids (White 2000); these could be altered by diagenesis and biodegradation to form carboxylic acids. Hopanes have structures similar to biomolecules, such as ergosterol (found in yeast), and other steroids found in both prokaryotes and eukaryotes (Tissot and Welte 1978). Another example is β-carotane ($C_{40}H_{76}$), a saturated form of β-carotene ($C_{40}H_{56}$) (Figure 1.2), which is abundant in Albanian petroleum (Sinninghe Damsté and Koopmans 1997). Compounds such as this may be biodegraded by bacteria, thereby becoming a naphthenic acid component. Bacteria are also able to reduce aromatics to aliphatic compounds. *Syntrophus aciditrophicus* combined with *Methanospirillum hungatei* was found to reduce benzoate to cyclohexylcarboxylate (El Shahed et al. 2001).

High temperatures during catagenesis alter the biomolecules through isomerization, aromatization or hydrogenation, cyclization, and bond cleavage (Brooks

Table 1.1 Naphthenic acids concentrations from different sources (CONRAD 1998, Brient et al. 1995).

Crude oil source	Naphthenic acids (wt%)
Russia, Romania, Poland	0.2 (Romanian, waxy) to 3 (typical)
United States	0.03 (Pennsylvania) to 4 (San Joaquin valley)
Venezuela, Lagunillas	1.2
Canada, Athabasca	2
Egypt	0.0015 to 0.403

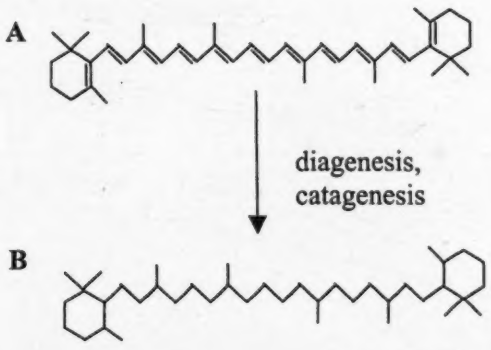


Figure 1.2 β-Carotene (A), a pigment commonly found in plants and microorganisms is the precursor of β-carotane (B), a major component of Albanian oil (Sinninghe Damsté and Koopmans 1997).

and Welte 1984, Sinninghe Damsté and Koopmans 1997). Transfer of one of the geminal methyls to another carbon during aromatization also occurs (Sinninghe Damsté and Koopmans 1997). Biodegradation of petroleum deposits may therefore produce carboxylic acids, which are similar but not identical to biomolecules. In the case of immature petroleum deposits, naphthenic acids may be present because of insufficient thermal alteration. Besides the difference in naphthenic acids structures due to disparity in biological origin, divergence in genesis would also add to the variation in naphthenic acids structure. Biodegraded petroleum would contain thermally altered biomolecules, which may not be found in immature petroleum.

1.1.3 Athabasca oil sands ore tailings at the Mildred Lake Settling Basin

In Table 1.1, the Athabasca oil sands have high naphthenic acids content (2% w/w). The Athabasca bitumen deposit is estimated to contain 1.7 to 2.5 billion barrels of bitumen, 300 million barrels of which is recoverable. This deposit is one of four bitumen deposits in Alberta, which includes the Peace River, Wabasca, and Cold Lake sites (Syncrude Canada Ltd. 2000).

Besides Alberta, Canada, other countries that have bitumen deposits include Venezuela, Madagascar, Albania, Romania, and Azerbaijan (Tiratsoo 1976). Unlike deposits in closed traps, which can be obtained using primary recovery, the Athabasca deposits are made up of bitumen deposited in pores of sand (Tiratsoo 1976). Some of the oil sands are exposed, as in the banks of Mildred Lake, while the rest have been covered by glacial drift (Tiratsoo 1976). Syncrude mines 155 million tonnes of oil sand per year, meeting 13% of Canada's petroleum needs. One barrel of Syncrude Sweet Blend (the final product made up of naphtha, light and heavy gas oils) is produced from two tonnes of oil sand. Oil sand from both the Mildred Lake and Aurora sites of the Syncrude lease are extracted ex-situ using steam, hot water, and elevated pH. Modern technologies allow extraction to proceed at 25 °C to 50 °C, instead of the 80 °C more commonly used. During extraction, sand and clay settle to the bottom of the vessels – this fraction is referred to as oil sands tailings or oil sands extraction wastewater. Bitumen floats to the top (primary froth). The middle layer is further extracted; this extract is combined with the primary froth. The combined bitumen extracts are upgraded to remove most of the sulfur, coked and hydrogenated to produce Syncrude Sweet Blend (Syncrude Canada Ltd. 2000).

Mildred Lake Settling Basin was created by Syncrude in 1978 to contain tailings water produced from the oil sands extraction (MacKinnon 1989). Syncrude has a zero waste discharge policy, which prevents them from releasing wastes into the environment. The settling basin has a volume of 300×10^6 m^3 (Leung et al. 2001). Tailings contain 50% to 55% solids and 0.5% bitumen (w/w) (MacKinnon 1989). The surface water, which results after the suspended solids settle, is re-used by the plant for extracting oil sands (MacKinnon 1989). This site will eventually be reclaimed, in accordance with

environmental regulations. Tailings pond waters contain 20 to 120 mg naphthenic acids/L (Holowenko et al. 2002). The lower concentrations are found in experimental ponds, which have not received fresh tailings for 7 to 11 years (Holowenko et al. 2002). Natural bodies of water in the oil sands area, such as Mildred Lake, contain 1.1 mg naphthenic acids/L (University of Waterloo 2001). The naphthenic acids are from the bitumen deposits in the area.

1.1.4 Commercial production and applications of naphthenic acids

Besides being part of oil extraction waste, naphthenic acids are processed from petroleum and are commercially available. AGS Chemicals and Products, Fluka (Sigma-Aldrich), JT-Baker, Kodak (Eastman Chemicals), Merichem, and Pfaltz and Bauer sell commercially prepared naphthenic acids. The composition and purity of the preparation depends on the petroleum source used. Crude preparations may contain phenolic and hydrocarbon compounds.

Petroleum fractions distilled between 200 °C to 370 °C are extracted with 2% to 10% NaOH to isolate the constituent naphthenic acids; the extracted naphthenic acids salts are acidified to change them back to their acid form (Brient et al. 1995). Naphthenic acids, naphthenic acids esters, and their metal salts have a variety of applications in industry as they: improve water resistance and adhesion of concrete; increase high pressure resistance of drilling oils; prevent foaming in jet fuel; prevent fungus growth in wood; preserve and act as flame retardants in fabric; increase insecticide solubility by acting as an emulsifier; catalyze rubber vulcanization; stabilize vinyl resins; catalyze production of alkyl and polyester resins (Brient et al. 1995). Naphthenic acids esters solidify at –43 °C, are not volatile, where 0.5% was in vapor form after 6 h at 100 °C, and lack color and odor (Mustafaev 1980). These properties make them suitable as stabilizers in vinyl resins. Naphthenic acids are surfactants, having a hydrophilic carboxylic acid group, and a hydrophobic carbon tail, which allows them to be used as emulsifiers. Industrial uses of naphthenic acids provide vectors for introducing these compounds to the environment. Although both oil sands mining and commercial production are sources

of naphthenic acids, the differences in their genesis and isolation could result in differences in their behavior in the environment.

1.2 Naphthenic acids in crude oil cause corrosion of processing equipment

Steel alloys resistant to corrosion by sulfide-containing compounds can be prone to corrosion by naphthenic acids (Kane and Cayard 1999). The process of naphthenic acids corrosion is not well understood, but it does involve the chelation of the metal ion by the carboxylate with the formation of hydrogen gas (Slavcheva et al. 1999). However, this process does not explain corrosion of alloys containing small (9%) amounts of Cr (Kane and Cayard 1999). High thoroughput rates, together with operating temperatures between 220 °C and 400 °C favor corrosion (Turnbull et al. 1998, Kane and Cayard 1999). Temperatures above 400 °C decompose naphthenic acids, forming a film that protects the alloy (Turnbull et al. 1998, Kane and Cayard 1999).

The type of naphthenic acids component also has an effect on corrosion. Corrosivity does increase with increasing crude oil total acid number (TAN). However, the efficiency of naphthenic acids corrosion not only depends on TAN but also on the availability of the carboxylic acid group to form metal complexes and the type of compounds present. Increasing alkyl chain lengths increases corrosivity when the chain has less than three methylene groups, after which corrosivity decreases (Turnbull et al. 1998).

A crude oil TAN greater than 0.5, or a refined oil TAN greater than 1.5 are considered corrosive (Kane and Cayard 1999). To mitigate this problem, alloys with aluminized steel, 18% Cr with 2.0 or 3.0% Mo may be used, corrosion inhibitors may be added to oil, and oil that has high TAN may also be diluted with oil that has a low TAN (Kane and Cayard 1999).

1.3 Biological effects of naphthenic acids

Naphthenic acids are believed to be the most toxic component of tailings water (CONRAD 1998, Madill et al. 2001) and refinery effluent (Wong et al. 1996).

Laboratory experiments show that naphthenic acids stimulate growth of bush bean (Wort and Patel 1970, Wort et al. 1973) but inhibit growth of aspen (Kamaluddin and Zwiazek 2002). They are toxic when taken up by mammals (Khanna et al. 1971, Rogers et al. 2002b). Aquatic life is the most susceptible to naphthenic acids toxicity because of prolonged and continuous exposure to these substances when dissolved in their habitat (Dokholyan and Magomedov 1981).

1.3.1 Naphthenic acids effect on plants

Foliar application of naphthenic acids to bush bean (*Phaseolus vulgaris*) (Wort and Patel 1970, Wort et al. 1973) stimulates their growth. On the other hand, growth of aspen (*Populus tremuloides*) was inhibited in medium containing 75, 150, and 300 mg naphthenic acids/L (Kamaluddin and Zwiazek 2002). The differences in the experimental results reported by Wort and Patel (1970), Wort et al. (1973), to that by Kamaluddin and Zwiazek (2002) may be due to differences in treatment (spraying leaves vs. growth in naphthenic acids contaminated medium) as well as differences in the types of plants used (bush bean, wheat, maize, sugar beet, and radish, vs. aspen).

Wort et al. (1973) measured indicators such as DNA and RNA synthesis, activity of nitrogen metabolism enzymes, amino acid and protein concentrations, as well as pod weight and yield to show that spraying plants with naphthenic acids stimulates their growth. Twenty millimolar potassium naphthenates in 0.3% Tween 20 was used. Bush bean protein concentrations increased in treated plants, compared to controls. Different amino acids were incorporated by controls and treated plants in different proportions. The greatest differences were found in levels of incorporation of arginine, lysine, glutamic acid, serine, and proline. Treated plants incorporated less arginine and lysine as well as more glutamic acid, serine and proline compared to controls. Treated plants also had higher concentrations of RNA compared to controls. There was an increase in the activity of enzymes involved in nitrogen metabolism as well as a 15.6% increase in the weight of bean pods produced per treated plant.

Part of a previous experiment (Wort and Patel 1970) compared the effects of different surrogate naphthenic acid compounds such as cyclohexanecarboxylic acid,

cylopentanecarboxylic acid, 3-cyclohexenecarboxylic acid, and cycloheptanecarboxylic acid. Only cyclohexanecarboxylic acid application provided greater green pod production ($P<0.05$). These observations indicate that naphthenic acids stimulate bush bean metabolism through the metabolic conversion of cyclohexanecarboxylic acid (Wort et al. 1973).

Growth medium containing naphthenic acids on the other hand, had a negative effect on aspen (Kamaluddin and Zwiazek 2002). Water movement through the plant was determined using parameters such as root hydraulic conductivity, root water flow, and stomatal conductance. Root hydraulic conductivity represents the mass of water flowing through an excised root system per unit change in applied water pressure, per second, per root volume ($kg/MPa*s*cm^3$). Stomatal conductance (mmol H_2O/m^2) is related to root hydraulic conductivity since the capillary pressure involved in non-metabolic water movement is possible because of the open stomata. The decrease in root hydraulic conductivity and stomatal conductance in treated plants show that aspen were subjected to osmotic stress.

Another parameter measured, root water flow (μL H_2O/min * root system), is metabolism dependent. It is measured by determining the volume of sap coming out of a severed stem per min, taking into account the number of root systems (i.e. entry points) when water is applied to the roots at constant pressure (0.3 MPa for 90 min). Root water flow was 52% to 57% lower through aspen after the roots were treated with naphthenic acids (Kamaluddin and Zwiazek 2002). Root respiration decreased to less than 70% of pre-treatment levels. The decrease in both root water flow and oxygen uptake rates, both show that naphthenic acids inhibit aspen metabolism (Kamaluddin and Zwiazek 2002).

According to Kamaluddin and Zwiazek (2002), metabolic effects are possible because 15 mg naphthenic acids/L (from the 150 mg/L treatment) to 21 mg naphthenic acids/L (from the 300 mg/L treatment) were found in xylem exudates. There was no detailed description as to how the naphthenic acids were differentiated from organic acids from the plants. Significant decreases in photosynthetic rates ($P<0.0001$) were observed, in conjunction with decreased chlorophyll production ($P<0.05$). Growth, as determined from leaf expansion after 5 weeks, was at least 50% less in aspen grown in naphthenic acids ($P<0.0001$). These data are further proof of the metabolic effects of naphthenic

acids on aspen. According to the investigators, naphthenic acids inhibited metabolic processes, and this affected water transport, and leaf growth. Naphthenic acids may also act like hydrocarbons that disrupt cell membranes (Kamaluddin and Zwiazek 2002).

From the studies of Wort and Patel (1970), Wort et al. (1973), and Kamaluddin and Zwiazek (2002), a generalization about the effects of naphthenic acids to plant life cannot be drawn. The major mechanism of metabolic inhibition by naphthenic acids was the decrease in water flow (Kamaluddin and Zwiazek 2002). Since Wort and Patel (1970), and Wort et al. (1973) did not expose plant roots to naphthenic acids, water flow would not be affected in their experiments. Differences in physiology between bush bean and aspen would also play a role in the differences in response to naphthenic acids.

1.3.2 Dissolved naphthenic acids are toxic to fish

The water solubility of naphthenic acids makes aquatic organisms more susceptible to their toxic effects. This is because organisms living in water have increased contact with naphthenic acids if their habitat is contaminated. Dokholyan and Magomedov (1984) determined the minimum naphthenic acids concentrations required to produce toxic effects on fish. The effect of species and age to naphthenic acids concentrations, which would produce toxic effects, were also considered.

Dokholyan and Magomedov (1984) studied acute toxicity by exposing fish species to 12 to 100 mg naphthenic acids/L for 10 days. Fifty percent mortality was observed in 2 month old chum salmon exposed to 25 mg naphthenic acids/L; 2 year old roach and Caspian round goby exposed to 75 mg naphthenic acids/L; 2 month old kutum, roach fingerling, and 2 year old sturgeon exposed to 50 mg naphthenic acids/L. The acute toxicity experiment showed that age of the test organism plays a role in its tolerance towards naphthenic acids. For example, whereas 50% of roach fingerlings died when exposed to 50 mg naphthenic acids/L, 75 mg naphthenic acids/L was needed to kill the same proportion of the 2 year old roach. Toxic effects of chronic, low level exposure to naphthenic acids was also determined. The most sensitive test organism was 2 month old chum salmon, for which a 60 day exposure to 3 mg naphthenic acids/L was 100% lethal. The effects of chronic exposure were also age dependent. Roach fingerlings exposed to

12 mg naphthenic acids/L for 45 day experienced 62 ± 3.8% mortality, whereas 2 year old roach experienced only 25 ± 2.9% mortality.

Dokholyan and Magomedov (1984) believed that estimates of lethality were insufficient for determining naphthenic acids toxicity. Other tests included measuring physiological and biochemical parameters such as leukocyte and glycogen levels. After 10 days exposure, 10% to 30% increases in leukocyte counts were observed in fish exposed to 0.5 to 5 mg naphthenic acids/L. This increase was followed by a 15% and 35% decrease in leukocyte counts in fish exposed to 1 to 5 mg naphthenic acids/L. Glycogen levels decreased to 50% of the control after 6 days exposure to 5 mg naphthenic acids/L. However, low naphthenic acids concentrations of 0.5 mg/L and 1 mg/L increased glycogen production.

A study at the University of Waterloo (2001) showed that naphthenic acids act as teratogens in Japanese medaka and yellow perch embryo. It was found that solutions containing 16 to 71 mg/L of the tailings water fraction, believed to contain naphthenic acids, resulted in toxic effects similar to concentrations of 2 to 5 mg/L of an unnamed commercial naphthenic acids solution. Toxic effects for Japanese medaka included decreased embryo survival, hatching success, hatchling length, increased instances of deformity, increased incubation time prior to hatching, and unsuccessful inflation of the swimming bladder. The embryo deformities for the medaka included misshapen heart, spine, and pericardial edema. Yellow perch embryos also showed spinal curvature, misshapen head, and shorter tails.

From the mortality, biochemical and physiological experiments of Dokholyan and Magomedov (1984), 0.5 mg naphthenic acids/L produces the least physiological and biochemical change in fish. There are other aquatic organisms that are more sensitive to naphthenic acids than fish. Among those tested, *Nephargoides maeoticus* (a zooplankton) can only tolerate naphthenic acids concentrations up to 0.15 mg/L (Dokholyan and Magomedov 1984). Dokholyan and Magomedov (1984) suggested 0.15 mg/L as the maximum allowable concentration of naphthenic acids in waters. This limit is unrealistic for the Athabasca oil sands region since the bitumen deposits naturally found in this region results in environmental naphthenic acid concentrations of 1.1 mg/L (in Mildred Lake, University of Waterloo 2001).

1.3.3 Dissolved naphthenic acids are toxic to rats

The effects of naphthenic acids on mammals have been studied using rats as model organisms. One study was by Khanna et al. (1971). A more recent study was done by Rogers et al. (2002b). They considered the effects of both acute and low-level chronic doses of naphthenic acids. Both groups have shown that naphthenic acids are toxic to rats.

In the study done by Khanna et al. (1971), 20 male albino rats, weighing 50 g each, were given ten 1 mL doses of 1% potassium naphthenates salts. Another set of 20 rats with similar attributes was treated as controls. Thus pairwise statistical comparisons were done. The naphthenic acids were injected into the area surrounding the abdominal cavity every other day for 21 days. There was no difference in the rate of weight gain between control and treated rats. Treated rats had elevated keto-steroid levels ($P<0.05$), red blood cells ($P<0.01$), and liver glycogen ($P<0.01$) compared to the controls. There was however, no significant difference in liver weight in the control and treated animals. Histological analysis of the testes in treated animals did show excessive cell growth. These findings were believed to suggest that naphthenic acids act like hormones. Hormonal effects, together with increased red blood cell and glycogen production may affect rate of maturity and reproduction of animals (Khanna et al. 1971).

Rogers et al. (2002b) did a more detailed study to determine the toxic effects of naphthenic acids on Wistar rats. Male and female rats were fed a single, high concentration dose of 3, 30, or 300 mg naphthenic acids/kg. These concentrations represent 0.5, 5, and 50 times the amount of naphthenic acids that animals are expected to be exposed to if they were in a highly contaminated environment. This test was done to determine the acute toxicity of naphthenic acids. Subjects in the acute toxicity study, which received 300 mg/kg naphthenic acids experienced toxic effects. These were manifested in the form of organ damage and increased organ weight ($P<0.05$ on a percent total body weight basis). In particular, females receiving naphthenic acids had heavier spleen and ovaries compared to controls. Males receiving naphthenic acids had heavier hearts compared to controls. The most pronounced difference in organ weight was in the

testes, where treated male testes were heavier than the controls. Liver and heart damage were more prevalent in females, while males experienced more brain hemorrhages.

The second experiment, which tests the effects of prolonged, subchronic intake of naphthenic acids by small mammals involved only female rats. The test rats were given 0.6, 6, or 60 mg naphthenic acids/kg, 5 days a week, for 90 days. This represents 0.1, 1, and 10 times the expected dosage, assuming a tailings naphthenic acids concentration of 100 mg/L. In the subchronic study, 25% of rats receiving 60 mg/kg and 17% of rats receiving 6 mg/kg naphthenic acids experienced severe seizures. The most pronounced increase in organ weight was found in the 60 mg/kg group, whose livers were 36% heavier than the controls. Their kidneys, and brain were also significantly ($P < 0.05$) heavier. For the first 11 days, the 60 mg/kg group consumed less food than the other groups, although food consumption became normal thereafter. This group also exhibited altered blood plasma biochemistry. There was a 43% decrease in cholesterol levels, and a 30% increase in amylase activity. The 60 mg/kg group also showed increased liver glycogen storage. There was however, no difference in the red blood cell production between treated subjects and controls.

Rogers et al. (2002b) believed that the liver was the primary target of naphthenic acids toxicity. This was manifested as liver damage and a 36% increase in liver weight in the acute experiments, and increased glycogen storage in the subchronic experiments. In both the acute and subchronic experiments, the rats were given the naphthenic acids solution orally. Controls received tap water. Observed toxic effects were dependent on naphthenic acids concentration, dosing (acute vs. subchronic), and in the case of acute dosage experiments, gender.

There were some differences between the observations by Khanna et al. (1971) and Rogers et al. (2002b). Whereas Khanna et al. (1971) found increased red blood cell production and no difference in liver weights when treated and controls were compared, Rogers et al. (2002b) observed the opposite. This may be due to differences in techniques, such as exposure route (injection through the stomach lining, vs. oral), and measurement methods. However, both Khanna et al. (1971) and Rogers et al. (2002b) observed elevated liver glycogen production and excessive testicular tissue growth in animals receiving naphthenic acids. Dokholyan and Magomedov (1984) also observed

increased glycogen in fish receiving low naphthenic acids concentrations. In addition to that, Khanna et al. (1971) found elevated keto-steroid production; whereas Rogers et al. (2002b) observed a decrease in blood plasma cholesterol levels. Keto-steroid and cholesterol levels, as well as testicular abnormalities are related since cholesterol is a precursor in steroid production, which includes androgens (Voet and Voet 1995). The conclusions drawn by the two groups are different in that Khanna et al. (1971), like Kamaluddin and Zwiazek (2002), believe that naphthenic acids act like hormones or hormone precursors. Rogers et al. (2002b) on the other hand, suggest that the physiological response results from induction of liver stress proteins; hence the liver is the target of naphthenic acids toxicity.

Considering the complexity of naphthenic acids mixtures, they may have multiple targets *in vivo*. As outlined in the discussions on the sources and structures of naphthenic acids, these compounds are borne from biomolecules, and in some cases may still retain aspects of their parent structure. The actual mechanism of naphthenic acids toxicity to rats is unknown.

1.4 Microtox™ acute toxicity assay

One of the major concerns regarding naphthenic acids is its toxicity. This is especially important when considering their presence in oil sands tailings waters. Toxicity assays that make use of complex eukaryotic organisms such as mice and fish are very expensive and consume much space and time. This inhibits processing large quantities of samples. Toxicity assays which use prokaryotes, such as Microtox™, are a possible solution.

1.4.1 The Microtox™ toxicity assay

Vibrio fischeri strain NRRL-B-11177, a luminescent bacterium, is the test organism used in the Microtox™ toxicity assay (Azure Environmental 2003). The method is quicker, easier and less expensive than other toxicity assays (Kaiser and Esterby 1991). Toxicity is expressed as the inhibitory concentration of the compound

responsible for x decrease in luminescence IC_x, (where x is the percent decrease in *V. fischeri* luminescence). A 50% decrease is referred to as the IC_{50}, whereas a 20% decrease is referred to as the IC_{20}. All Microtox™ protocols make use of dilution series to determine IC values. The two most commonly used protocols are the Basic and the 100% protocols. Tarkpea and Hansson (1989) have compared the two protocols.

Both the Basic and 100% assays make use of four dilutions of the toxicant, and a control. The Basic assay consists of 90, 45, 22.5, 11.25, and 0 %v/v dilutions of a toxicant. Dilutions are made using a salt solution specifically formulated by Microtox™. A second 1:1 dilution series is performed, making the final %v/v concentrations in the Basic protocol 45, 22.5, 11.25, 5.6, and 0 %v/v. The 100% protocol on the other hand makes use of the first dilution series only. This result in final %v/v concentrations of 90, 45, 22.5, 11.25, and 0 %v/v.

Besides the differences in dilution series, and therefore upper concentration limits between the Basic and 100% protocols, the calculations involved also differ. IC values are determined by plotting the change in light output against sample concentration (in %v/v) (Microbics 1991). Because of the second series of dilutions involved in the Basic protocol, a correction can be done. This takes into account the natural decrease in luminescence over the incubation time by using the 0 %v/v dilution. The luminescence is measured prior to (I_o), and after (I_t) toxicant addition to *V. fischeri* suspensions. The equation, which determines the loss in light ouput (Γ_B) therefore becomes:

$$\Gamma_B = \left[\frac{\left[\frac{I_{tc}}{I_{oc}} \right] (I_o)}{I_t} \right] - 1$$

where:

Γ_B = loss in light output

I_{tc} = light output of control after incubation time

I_{oc} = light output of control at time 0

I_o = light output of sample at time 0

I_t = light output of sample after incubation time

Equation 1.1 (Microbics 1991)

The 100% test, unlike the Basic protocol, does not have a correction embedded in the equation. Here, light output from each dilution is measured after the incubation time (usually 5 or 15 min for both Basic and 100% protocol). Light output (Γ) is calculated by subtracting "1" from the quotient of the control (I_c) and sample (I_s) light output, for each dilution (Equation 1.2).

$$\Gamma_{100} = \left[\frac{I_c}{I_s} \right] - 1$$

where:

Γ_{100} = light output

I_c = light output of control after incubation time

I_s = light output of sample after incubation time

Equation 1.2 (Microbics 1991)

By definition, IC_{50} is the concentration where the light ouput of the sample is 0.5 of the control, making $\Gamma = 1$. IC_{20} is the concentration where the light output of the sample is 0.8 of the control, making $\Gamma = 0.25$. Because the Basic protocol takes into account changes in luminescence of *V. fischeri* over time, it is less sensitive to experimental variations than the 100% protocol.

Effluents from textile, food, metal, pharmaceutical, detergent, and mechanical industries were used to determine the agreement between the two methods (Tarkpea and Hansson 1989). Results from the 100% and Basic tests were found to be significantly different from each other, by less than an order of magnitude. The 100% protocol considered the samples less toxic, compared to the results from the Basic protocol, in 25 out of 28 samples ($P < 0.001$). The confidence interval (CI) for the 100% test was 10 times broader, compared to the Basic protocol, making the 100% test less accurate.

Tarkpea and Hansson (1989) analyzed 15 samples, in duplicate. After 3 months, 13 of these were analyzed a second time, in duplicate. There were therefore 56 analyses done using the Basic protocol and 56 analyses using the 100% protocol. The accuracy of the two protocols was compared using the 95% CI lengths. Samples analyzed within 3 months were treated separately. The source of each sample was not indicated; alphanumeric codes were used to distinguish individual samples. The difference in 95%

CI length between the Basic protocol was compared to that of the 100% protocol for the first set of analyses, as well as those done after 3 months. Here, a value closer to 1 means that the CI difference is similar for both analyses times. These values ranged between 0.95 for sample A1 (100% protocol was 25.5 times wider on the first day and 24.2 times wider after 3 months) to 0.02 for sample G2 (100% protocol was 16.2 times wider on day 1 and 3.8 times wider after 3 months). On average, the 95% CI of the 100% protocol was ten times wider than that of the Basic protocol. The 95% CI for the 100% protocol in fact, was 46 (95% CI for 100% = 0.92, for Basic = 0.02) to 0.4 (95% CI for 100% = 0.63, Basic = 1.72) times wider than the Basic protocol. Seventeen out of 28 CI comparisons showed that the 100% protocol was 1.2 to 7.5 times wider than the Basic protocol.

Although the 100% protocol has a wider concentration range, it also has a wider CI and is therefore less precise. Only IC_{50} values can be determined using the 100% protocol (Tarkpea and Hansson 1989). The Basic protocol is more commonly used. It generally has a shorter CI because the I_o readings for each dilution are done prior to adding the toxicant, unlike the 100% protocol. The Basic protocol is therefore used in all Microtox™ analyses of naphthenic acids toxicity discussed hereafter.

1.4.2 Comparison of other toxicity assays to Microtox™

Microtox™ is one of many bioassays used to determine the toxicity of environmental contaminants. Besides *V. fischeri*, test organisms include fish such as rainbow trout, fathead minnow, goldorfe; zooplankton such as *Daphnia magna*; other organisms such as ciliates, algae, and mammals (rats and mice) are also used. A review by Munkittrick et al. (1991) cited that Microtox™ has one-third the variability of other assays (*Daphnia*, rainbow trout, fathead minnow). This was based on 129 assays performed by the Canadian Petroleum Association, using waste drilling fluids. Kaiser and Esterby (1991) made statistical comparisons between fathead minnow, goldorfe, *Daphnia*, *Tetrahymena pyriformis* (ciliate), *Scenedesmus quadricauda* (algae), octanol-water partition coefficient (K_{ow}), Norway rat (acute oral dose), and Microtox™ using data from literature to determine comparability of these assays. Toxic compounds were grouped according to chemical classes. They found that an increase in the K_{ow} is strongly

correlated to toxicity increase, except for the rat assays. There was high correlation between Microtox™ and $\log K_{ow}$ ($R^2 = 0.985$) in compounds containing carboxylic acids.

For 2000 chemicals found in toxicity databases, correlation between rat, mouse and Microtox™ was dependent on the uptake route (Kaiser et al. 1994). Rat and mouse data had high correlation to each other for any given administration route (i.e. oral, intraperitoneal, intravenous). Intravenous application of the toxicant had the highest correlation with Microtox™ ($R = 0.73$ for rat, and 0.68 for mouse). The lack of correlation with the intraperitoneal and oral dose routes was believed to be due to metabolism of the toxicant (Kaiser and Esterby 1991). Since the mechanism of toxicity of naphthenic acids is mainly unclear, it is unknown whether complex organisms metabolize them.

Of more specific interest is a study done to determine differences in sensitivity between rainbow trout, *Daphnia*, and Microtox™ to tailings pond water toxicity (MacKinnon and Boerger 1986). The tailings water concentrations (%v/v) lethal to 50% of the trout or *Daphnia* population after 96 h exposure were defined to be the LC_{50}. The concentration (%v/v), which decreased *V. fischeri* luminescence by 50% over 15 min incubation, using the Basic assay was defined to be the IC_{50}. All three assays were compared. The tailings were found to be more toxic to *Daphnia* (2% v/v tailings water was lethal to 50% of the population) and trout (7% v/v tailings water) compared to Microtox™ (20% to 30% v/v tailings water IC_{50} after 15 min incubation). However, the Microtox™ IC_{20} (20% decrease in luminescence) was determined to be 10% v/v tailings water, and is more reproducible compared to the trout and *Daphnia* assays. Because of this, the Microtox™ IC_{20} was determined to be suitable to describing tailings pond water toxicity (MacKinnon and Boerger 1986).

These studies show that the Microtox™ acute toxicity assay is a useful and widely accepted tool for determining toxicity of organic compounds. For tailings water, it is more reproducible compared to other assays. However, differences in toxicant metabolism must also be considered. Microtox™ is a useful, widely available tool, but may not predict all toxic effects experienced by complex organisms.

1.4.3 Toxicity decrease measured using Microtox™

Biodegradation usually decreases the concentration of toxic compounds. This could then lead to a decrease in the toxicity of a contaminated site. Decreases in toxicity of oil refinery wastewaters as measured using Microtox™ followed polycyclic aromatic hydrocarbon and total organic carbon loss closely (Munkittrick 1991). Decreases in naphthenic acids toxicity as a result of decreases in concentrations have also been analyzed using Microtox™ (MacKinnon and Boerger 1986, Herman et al. 1994, Holowenko et al. 2002).

Among the experiments was the biodegradation of tailings water extracts in aerobic microcosms (Herman et al. 1994). Sixteen percent of the 50 mg carbon/L in the extract was released as CO_2 after 9 days aerobic incubation. This corresponded to decreased toxicity as measured using Microtox™ IC_{50}. Controls and time zero samples had IC_{50} values of around 25% v/v whereas biomineralized samples had IC_{50} values of about 50% v/v. Experiments on oil sands tailings water disposal sites have also been performed to better understand naphthenic acids behavior in the environment.

MacKinnon and Boerger (1986) analyzed toxicity as well as organic and inorganic component concentrations of tailings water from Mildred Lake Settling Basin, and other experimental ponds containing tailings water. Acidification of the sample, which would allow naphthenic acids adsorption onto sediment, was found to be the most effective means of decreasing toxicity. Two-year storage in 3 to 5 m deep, well-aerated pits was also shown to decrease tailings water toxicity, as shown using the Microtox™ IC_{20}. The time zero sample had an IC_{20} of 35% v/v, this decreased to a 100% v/v IC_{20} after 2 years. At 2 years, suspended solid concentrations had decreased to 15 mg/L, compared to 50 mg/L at time zero. Chemical oxygen demand (a measure of organic carbon concentration) also decreased to 20 mg/L, compared to 150 mg/L at time zero. A more recent study by Holowenko et al. (2002) also determined naphthenic acids concentration and toxicity from tailings water storage ponds. The sites included the Mildred Lake Settling Basin and an 11-year-old pond, containing tailings water (Holowenko et al. 2002). The study also found evidence that tailings water toxicity decreases with decreasing naphthenic acids concentrations. Tailings water from Mildred

Lake Settling Basin had concentrations of 49 mg/L, and Microtox™ IC_{20} of 7% v/v, whereas the 11-year sample had a naphthenic acids concentration of 24 mg/L and an IC_{20} of 100% v/v. The Microtox™ acute toxicity assay is a convenient method to determine changes in sample toxicity due to changes in naphthenic acids concentrations.

1.5 Methods used to analyze naphthenic acids concentrations

In studying the effects of naphthenic acids such as corrosion and toxicity, as well as its biodegradation, its concentrations must be measured. This is a challenge because of the complexity of the mixture. Other crude oil components, which are co-extracted with naphthenic acids, may also interfere with the determinations.

1.5.1 Methods used to analyze naphthenic acids concentrations

Naphthenic acids concentrations have been expressed in terms of TAN. This value is determined by titrating the sample with KOH, using either potentiometric (ASTM D664) or colorimetric (ASTM D974) detection (Brient et al. 1995, Drews 1998, Slavcheva et al. 1999). It is expressed in terms of mg KOH/g sample. In potentiometric analysis, either an inflection point, or a millivolt reading similar to the standard is determined to be the end point (Drews 1998). Colorimetric assays make use of an indicator which changes color at the desired pH. Titration measures TAN, it is therefore not highly selective for naphthenic acids. Other methods have been developed to help determine naphthenic acids concentrations (Table 1.2).

Yu and Green (1989) analyzed model naphthenic acid compounds and a crude oil sample. Infrared (IR) absorption of the carbonyl group of the fluoroester was determined at ~1800 cm^{-1}. Reproducibility of the method was determined by comparing the molar absorptivity coefficient (ϵ) of each analysis. This value was reproducible, with 2% to 5% relative standard deviation. The carbonyl group from the product does not experience as much interference as its underivatized form. The method was, however, labor and time consuming. Samples undergo esterification for 15 min, followed by a 30 min acylation step. Excess reagents interfere with the sample and must be extracted twice into aqueous

Table 1.2 Summary of methods, other than titration, used to determine naphthenic acids concentrations.

Method	Reference	Description
IR of fluoroesters	Yu and Green 1989	Quantitative Measures carbonyl absorption of fluoroester derivative (~1800 cm^{-1})
GC-FID	Herman et al. 1994	Methyl esters Semi-quantitative Compares relative hump areas of sterile and biodegraded naphthenic acids to stearic acid (internal standards)
	Jones et al. 2001	Methyl esters Quantitative Elutes between 5 and 80 min Eight crude oil solid phase extracts compared to 1-adamantanecarboxylid acid and 5β-cholanic acid (internal standards)
FT-IR	Jivraj et al. 1995 Holowenko et al. 2001 Rogers et al. 2002a	Underivatized Quantitative Measures both monomer and dimer carbonyl peak heights at 1743 and 1706 cm^{-1} and compares them to a series of standards
Electrospray ionization-MS	Headley et al. 2002 Morales-Izquierdo 1999 Rogers et al. 2002a	Underivatized Quantitative Measures naphthenic acids concentrations after extraction and concentration

buffer. The reaction derivatizes both hydroxyl and carboxyl groups, and so is not especially selective for naphthenic acids.

More recently, Fourier transform IR (FT-IR) spectrometry has been used to determine naphthenic acids concentrations (Jivraj et al. 1995, Holowenko et al. 2001, Rogers et al. 2002a). The method involves extracting naphthenic acids into organic solvent and measuring the carbonyl group absorbance. The absorbances of the monomeric and dimeric forms of the carbonyl are summed. The sample is exposed to the IR range simultaneously and could therefore be scanned repeatedly within a short period of time (Silverstein and Webster 1998). Averaging multiple scans increases the signal to noise ratio (since baseline noise is random), making concentration measurements more accurate (Skoog and Leary 1992). This method is less time consuming than that of Yu and Green (1989). However, it may overestimate naphthenic acids concentrations because of other carbonyl groups which may be present (Yu and Green 1989).

Naphthenic acids have also been derivatized to form their methyl esters and analyzed using GC with flame ionization detection (FID) (Herman et al. 1994, Jones et al. 2001). Herman et al. (1994) extracted the naphthenic acids from laboratory cultures. Here, the naphthenic acid methyl ester "hump" (i.e. unresolved complex mixture) was integrated and compared to a stearic acid internal standard. Jones et al. (2001) extracted naphthenic acids from crude oil using a quaternary amine solid phase extraction ion exchange column. Carboxylic acid derivatives were formed by reacting the sample with either BF_3 in MeOH or diazomethane. The methylated carboxylic acids were then extracted using a silica column. Samples derivatized with diazomethane had higher recoveries. The reagent is however, potentially explosive. Relative standard deviations for three to four replicate analyses of BF_3/MeOH methylated crude oil acids ranged from 7.5% to 18.0%.

Mass spectrometry (MS) using electrospray ionization can be used to determine naphthenic acids concentrations (Morales-Izquierdo 1999, Headley et al. 2002, Rogers et al. 2002a). The value for a tailings extract sample was around 97% of that estimated using FT-IR (Rogers et al. 2002a). Rogers et al. (2002a) and Morales-Izquierdo (1999) extracted samples using liquid-liquid extraction, with dichloromethane as the organic solvent. Headley et al. (2002) used solid phase extraction. Headley et al. (2002) found

R^2 of 0.9998, for the calibrations curves and detection limits of 0.01 mg/L for both a Fluka commercial preparation, and Syncrude oil sand acidic fraction. Morales-Izquierdo (1999) found R values of 0.91 to 0.99 for calibration curves.

1.5.2 Fatty acid concentrations determined by HPLC

The carboxylic acid group of naphthenic acids has been used in determining their concentrations. Although the aliphatic portion of naphthenic acids differs from that of fatty acids, both contain the carboxyl group which is useful in determining concentrations. Fatty acids and other compounds containing carboxylic acids are ubiquitous, which explains the great interest in measuring their concentrations. Brondz (2002) goes into the history of chromatography, classifications of fatty acids, and methods of determining their concentrations by GC and HPLC.

Reaction of fatty acids with chromophores is necessary because of their low extinction coefficient. A solution in ethanol for example, has an ϵ value of 50, at 210 nm, compared to at least 1000 for compounds with conjugated double bonds (Brondz 2002), making carbonyl absorption readings insensitive indicators of carbonyl concentration (from the Beer-Lambert equation). Table 1.3 gives some examples of derivatives formed to detect fatty acids. Some derivatives, such as those of 9-anthryldiazomethane (Kargas et al. 1990), and 1-pirenyldiazomethane (Yan et al. 1999) can be analyzed by both UV-visible and fluorescence spectroscopy. Phenacyl ester formation, first developed by Durst et al. (1975) is commonly used. The original method made use of 18-crown ether as a catalyst; later variations (Wood and Lee 1983) use triethanolamine. The reactions are done in organic solvent. A sample in aqueous solution must therefore first be extracted prior to analysis.

1.5.3 Derivatization of carboxylic acids with 2-nitrophenylhydrazine

A method was developed in the 1980s to produce 2-nitrophenylhydrazine (NPH) derivatives of fatty acids (Miwa et al. 1985). The reaction scheme is shown in Figure 1.3, and is based on peptide synthesis chemistry as shown by McMurry (1995). The coupling

Table 1.3 Examples of derivatives commonly used to determine fatty acid concentrations.

Derivative	Reference
Phenacyl	Durst et al.1975
Isopropylidene hydrazides	Agrawal and Schulte 1983
p-Nitrobenzyl	Bandi and Reynolds 1985
9-Anthryldiazomethane	Kargas et al.1990
Monodansylcadaverine	Lee et al.1990
1-Pirenyldiazomethane	Yan et al.1999

agent normally used in peptide synthesis, dicyclohexylcarbodiimide, is insoluble in aqueous solvent. Replacement with 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC) allows the reaction to proceed in aqueous solution. This method has been extensively characterized and optimized in terms of reagent concentrations, and pH (Miwa et al.1985, Miwa and Yamamoto 1987, Miwa 2000).

The product has two absorption maxima, at 230 and 400 nm, when pH is less than 8.5 (Miwa et al. 1985). The 400 nm wavelength is recommended as the detection wavelength, since it is subject to less interference than 230 nm (Miwa et al. 1985). This method has been used to detect short (Miwa et al. 1985), medium (Miwa and Yamamoto 1986), and long chain (Miwa and Yamamoto 1986, 1987) fatty acids. The absorption maxima are pH dependent, and change to 550 nm at pH 12 (Miwa et al. 1985). Detection at 550 nm has also been used to determine concentrations of non-ionic surfactants (Kondoh et al. 1991). It involves on-line reaction after the fatty acids have been resolved by HPLC.

Derivatization of carboxylic acids with NPH could therefore be applied to the analysis of naphthenic acids. The method developed by Miwa et al. (1985) would allow direct analysis of naphthenic acids in aqueous solutions without extraction into organic solvent. The availability of HPLC and UV-Visible spectrometry makes this method widely accessible.

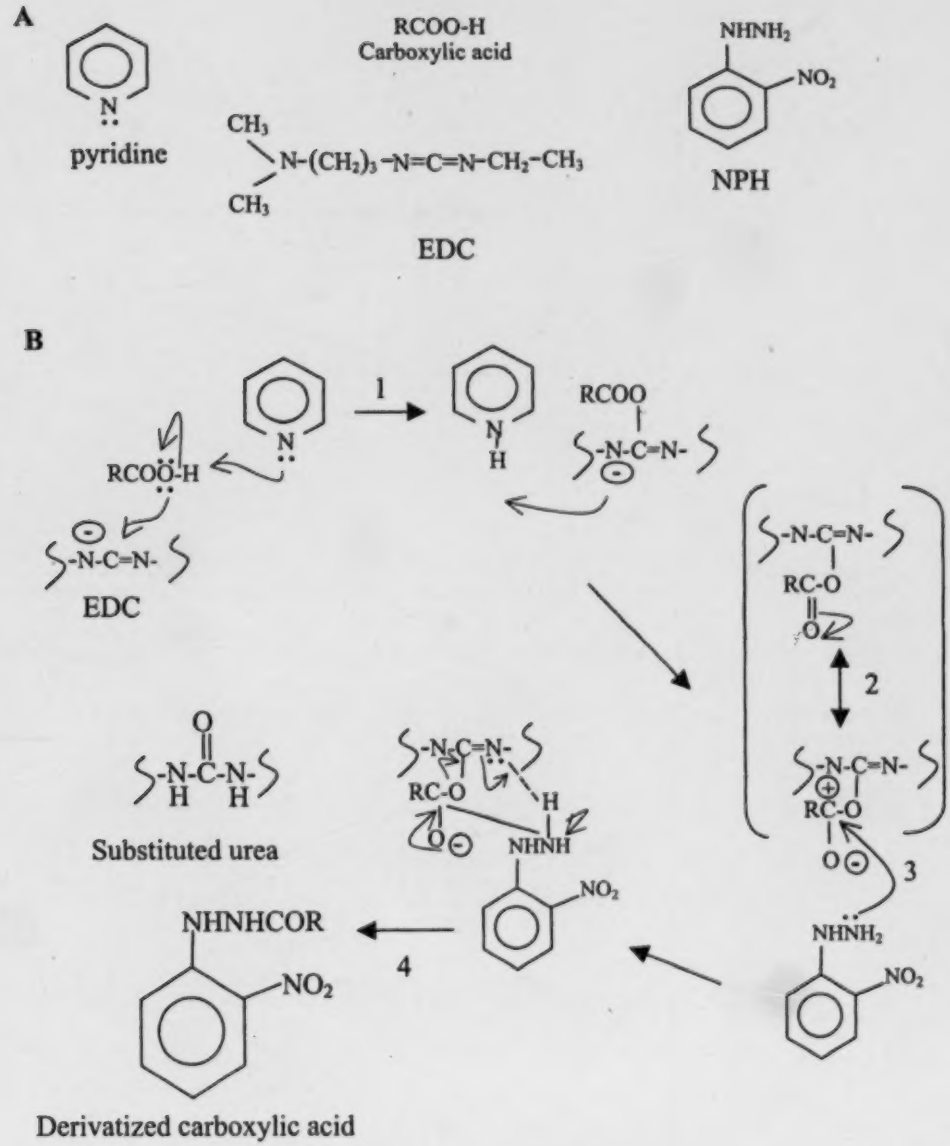


Figure 1.3 Reaction mechanism for NPH derivative formation. (A) compounds involved in the reaction, (B) reaction mechanism. The following steps are shown: (1) pyridine catalyzes attachment of carboxyl and carbodiimide portion of EDC to generate a reactive acylating agent, (2) carboxyl resonance, (3) NPH nucleophilic attack on carbonyl carbon, (4) formation of a by-product (substituted urea) and the carboxylic acid derivative (McMurry 1995).

1.6 MS used to determine naphthenic acids composition

Determining the total naphthenic acids concentration may not be sufficient to explain their corrosive and toxic effects. Current information on the structures and compositions of naphthenic acids arose from MS studies.

1.6.1 Introduction to terms used in MS

Silverstein and Webster (1998) give a brief introduction to MS. All mass spectrometers give the mass of a charged molecule based on its behavior as it passes through a magnetic field. Molecules can be ionized by several methods: Electron impact ionization (EI) produces ions by bombarding the sample with electrons having enough energy to remove an electron from the sample. Chemical ionization produces secondary ions where a reagent gas reacts with the sample, thereby producing an ion. Except for atmospheric pressure chemical ionization, chemical ionization generally occurs at a pressure of 1 torr. Electrospray ionization is generally used for water-soluble compounds with inherent charge. Here, a fine mist (sample in aqueous solvent) is dried into individual ions by a nebulizer as it escapes a needle. In fast atom bombardment, the sample (which is in a volatile, viscous matrix) is ionized by a beam of xenon or argon. Chemical ionization, fast atom bombardment, and electrospray ionization are considered soft ionization techniques because they do not induce fragmentation. The fragmentation patterns observed after EI is characteristic for each type of compound and may help identify unknowns.

Tandem MS is essentially two mass spectrometers coupled to each other. The first ionization event produces the primary ion, while the second ionization event produces the secondary ion. The mass losses which are involved in producing the secondary ion can be used to identify the presence of functional groups.

1.6.2 Summary of ionization techniques used to characterize naphthenic acids

The MS methods used to study naphthenic acids are summarized in Table 1.4.

Most of the studies on these compounds have used EI (Seifert and Teeter 1969, Seifert et al. 1969, Lebedevskaya et al. 1977, Green et al. 1994, St. John et al. 1998, Jones et al. 2001, Tomczyk and Winans 2001, Holowenko et al. 2002). This method has been used mainly because it was readily available. All EI experiments involved derivatizing the naphthenic acids prior to analysis. Changing the acids to neutral species decreases their boiling point, making them more volatile, and thus available for EI analysis. The experiments performed by Seifert and Teeter (1969), and Seifert et al. (1969) involved time consuming separations such as thin layer chromatography (Seifert and Teeter 1969), and silica gel chromatography, followed by gel permeation chromatography (Seifert et al. 1969). Extractions and purification steps were necessary because EI does not discriminate between naphthenic acids and hydrocarbons. Another complexity is that EI imparts more energy than necessary to ionize the sample. The excess energy is used to fragment the ions (Silverstein and Webster 1998). Although this effect is useful in identifying pure compounds, it is problematic when analyzing naphthenic acids. The fragment ions cannot be differentiated from molecular ions because the complexity of the naphthenic acids mixture results in ion overlaps. This makes it difficult to determine naphthenic acids composition (Dzidic et al. 1988, Qian et al. 2001, Barrow et al. 2003).

Derivatized naphthenic acids can be introduced to the mass spectrometer slowly by distillation off the probe, or by using GC. Distilling the acids involves increasing the probe temperature, and averaging the readings over the temperature range (Seifert et al. 1969, Tomczyk and Winans 2001). GC has been used to slowly introduce samples to the MS (Green et al. 1994, St. John et al. 1998, Holowenko et al. 2002). Different components of the naphthenic acids mixture should have different boiling points because of differences in saturation and number of carbons. Different fractions would therefore be available for ionization at different GC temperatures. Although naphthenic acids cannot be completely resolved by either method, the slow sample introduction results in more accurate ion monitoring.

Fast atom bombardment has been characterized by Fan (1991). He compared ion distributions obtained using chemical ionization by looking at results of Dzidic et al. (1988) to those experimentally determined using fast atom bombardment (Fan 1991). A wider range of formula weights was identified with fast atom bombardment compared to

Table 1.4 Ion sources used to study naphthenic acids by MS.

Method	Reference	Description
EI with probe	Seifert and Teeter 1969	Fluoroalcohol derivatives.
	Seifert et al. 1969	Hydrocarbon derivatives. Three step reaction (57 h). Distill sample from probe by increasing temperature from about 60 °C to 300 °C.
	Lebedevskaya et al. 1977	Trimethylsilyl ester derivatives.
	Tomczyk and Winans 2001	Methyl ester derivatives. Distill sample off the probe by increasing the temperature from 200 °C to 400 °C.
EI with GC	Green et al. 1994	Fluoroester derivatives. Unlike methyl esters, interference were away from ions of interest.
	St. John et al. 1998	*t*-Butyldimethylsilyl ester derivatives. Results are similar to Dzidic et al. (1988). Difficult to identify acids with less than 9 carbons.
Fast atom bombardment	Fan 1991	Xenon ionizes sample which is in triethanolamine matrix. Detected a wider range of ions compared to chemical ionization.
Chemical ionization	Dzidic et al. 1988	Fluoride ion ionization. Distill off probe by increasing temperature from 70 °C to 300 °C.

Continued on next page.

	Hsu et al. 2000	Performed under atmospheric pressure. Acetonitrile as solvent. Selectively ionizes naphthenic acids. Superior sensitivity to electrospray ionization.
Negative ion electrospray ionization	Morales-Izquierdo 1999	Used 5% ammonia in 1:1 water:methanol as solvent. Analyzed commercial preparations, and extracts from tailings ponds in the Athabasca oil sands.
	Rudzinski et al. 2002	Used methanol containing 0.5% NH_3 as solvent. Superior sensitivity to atmospheric pressure chemical ionization.
	Lo et al. 2003	Analyzed surrogate, commercial and Athabasca oil sands naphthenic acids.
Negative ion electrospray ionization with FT ion cyclotron resonance	Qian et al. 2001	Ultrahigh resolution. Selectively ionized naphthenic acids in crude oil.
	Barrow et al. 2003	Efficiently produces ions. Ultrahigh resolution. Selectively ionize naphthenic acids.
Negative ion electrospray ionization with high-field asymmetric waveform ion mobility spectrometry	Gabryelski and Froese 2003	Can be coupled to either quadrupole or time of flight spectrometers. Extraction and derivatization unnecessary. Able to detect ions (m/z 373, 417 and 461) which are not seen with GC-MS.

chemical ionization. Fast atom bombardment detected compounds with up to 50 carbons, whereas chemical ionization can only detect compounds with up to 34 carbons. Hsu et al. (2000) compared their fast atom bombardment experiment results with those of Fan (1991); the results were found to be consistent. However, although fast atom bombardment was shown to detect formula weights greater than 340 (approximately 22 carbons), these are mainly non-covalently associated ions (Hsu et al. 2000).

Chemical ionization methods have been used by Dzidic et al. (1988) and Hsu et al. (2000) for naphthenic acids analysis. Hsu et al. (2000) compared ion profiles of samples ionized using fast atom bombardment, chemical ionization, atmospheric pressure chemical ionization, and electrospray ionization. Chemical ionization and atmospheric pressure chemical ionization selectively ionizes naphthenic acids (Dzidic et al. 1988, Hsu et al. 2000). Dzidic et al. (1988) used fluoride ions to deprotonate the naphthenic acids. Hsu et al. (2000), on the other hand, used acetonitrile; it forms CH_2CN^-, which deprotonates the naphthenic acids. Chemical ionization was found to be biased towards lower molecular weight compounds, possibly because of inefficient ionization, decarboxylations, and low volatility of high formula weight compounds (Hsu et al. 2000).

Negative ion electrospray ionization is capable of detecting the ionic form of naphthenic acids, which predominates under basic conditions. Commercial naphthenic acids from Kodak and Pfaltz and Bauer, as well as naphthenic acids extracted from tailings waters have been analyzed by Morales-Izquierdo (1999). Both the ion distribution and total naphthenic acids concentrations can be determined using this method. Comparisons between atmospheric pressure chemical ionization and electrospray ionization were done by Hsu et al. (2000) and Rudzinski et al. (2002), with conflicting conclusions (Table 1.4). The groups used different solvents for electrospray ionization analyses, which resulted in different conclusions as to which method was more sensitive (Rudzinski et al. 2002). Electrospray ionization can be coupled to FT-ion cyclotron resonance MS, allowing ultrahigh resolution analyses, with minimum sample volume (Barrow et al. 2003, Qian et al. 2001).

A recently reported method which uses electrospray ionization uses high-field asymmetric waveform ion mobility spectrometry to separate ions, in place of a GC (Gabryelski and Froese 2003). The separation technique was coupled with quadrupole

and time of flight MS. It was possible for tandem MS to be performed. Unlike other MS methods, this has the advantage of pre-separation without the extraction and derivatization steps. Dilution of tailings pond waters to 1 mg naphthenic acids /L with methanol was the only preparatory step performed.

1.6.3 t-Butyldimethylsilyl esters of naphthenic acids

St. John et al. (1998) developed a method which uses *N*-methyl-*N*-(*t*-butyl-dimethylsilyl)trifluoroacetamide (MTBSTFA) to derivatize naphthenic acids to their *t*-butyldimethylsilyl ester (Table 1.4). It is a convenient method involving a 20 min reaction at 60 °C. This method was used by Holowenko et al. (2002) to determine naphthenic acids distributions in waters of several oil sands tailings ponds. Both the reaction to produce an ester, as well as the expected fragmentation for a carboxylic acid is shown in Figure 1.4. St. John et al. (1998) were able to characterize ions from surrogate naphthenic acids. They found that base peaks consist of the $[M+57]^+$ ions (where M is the mass of the underivatized naphthenic acid), which forms after the positively charged radical fragments (Figure 1.4, B). The *t*-butyl group is cleaved off, leaving a dimethylsilyl ester. The dimethylsilyl ester (base peak) can further fragment to form a positively charged dimethylsilyl alcohol with a mass to charge (m/z) ratio of 75 (Figure 1.4B, step 2, compound b), and a neutral specie not detected by the MS (Figure 1.4B, step 2, compound a).

Camphoric acid, a dicarboxylic acid, was also analyzed by St. John et al. (1998). The *t*-butyldimethylsilyl ester formed at both carboxylic acids. Introduction of the sample to the GC-MS however, shows that the major peak resulted from cleavage of only one *t*-butyl group, leaving a singly charged ion. Studies using pure compounds demonstrated the stability of the base peak (i.e. the $[M+57]^+$ peak). That ion could be used to determine the relative abundance of a naphthenic acid component from a mixture. St. John et al. (1998) found that the ion profile obtained with this method was similar to that from fluoride ion chemical ionization obtained by Dzidic et al. (1988). Analytical bias resulting from naphthenic acid formula weight or structure has however, not been extensively studied, unlike those done for fluoroalcohol esters (Green et al. 1994).

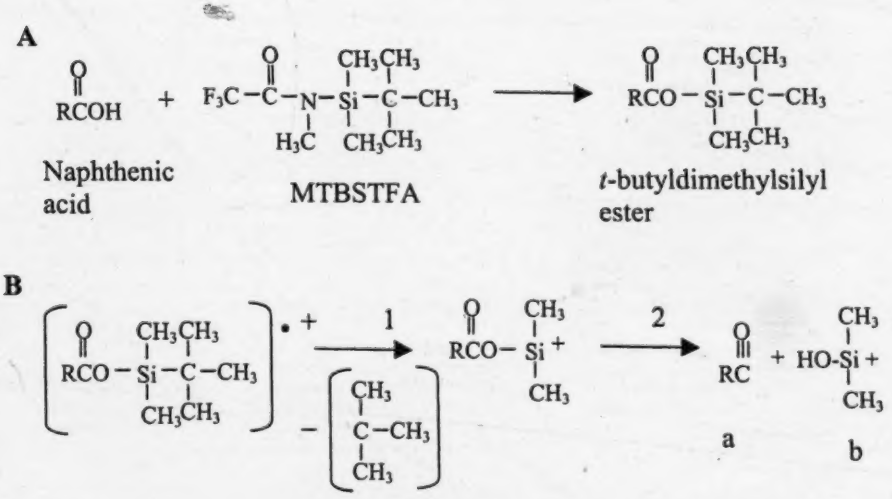


Figure 1.4 Reaction of a naphthenic acid with the derivatizing agent MTBSTFA to produce the *t*-butyldimethylsilyl ester (A). The ester is ionized after EI (B). Release of the *t*-butyl group results in the major ion, a positively charged dimethylsilyl ester (1) which can fragment further (2) resulting in a neutral fragment (a), and a positively charged dimethylsilyl alcohol (b) (St. John et al. 1998).

1.6.4 Applications and limits of commonly used MS methods

The naphthenic acids general formula and structural definition are used to assign the ions observed through MS to a particular carbon number and Z family combination. Holowenko et al. (2002) summarized the ions observed through EI-MS, analyzed using the method developed by St. John et al. (1998) as three-dimensional graphs with percent abundance in the vertical axis, carbon number as the x-axis, and Z family as the y-axis. The graphs make it easier to visualize the information obtained through MS. Variations of three-dimensional graphs were also generated by Rudzinski et al. (2002), and Tomczyk and Winans (2001).

Table 1.5 shows how the Z family and carbon numbers are assigned, given the naphthenic acids general formula $C_nH_{2n+Z}O_2$, and the stipulation that cyclic compounds are also substituted with an alkyl. Table 1.5 is equivalent to a coordinate system, where each cell contains the chemical formula and molecular weight of a naphthenic acid with

the corresponding carbon number, and Z family. Table 1.6 shows the same compounds as in Table 1.5, after derivatization, thereby becoming $[M+57]^+$ ions. Cells with a diagonal line across correspond to compounds which cannot be considered naphthenic acids because of structural constraints. This means that compounds with not enough carbon or hydrogen atoms to fulfill the predicted structure are excluded. For example, cyclopentanecarboxylate does not follow the definition of a naphthenic acid, and so the cell corresponding to C=6, Z=-2 is crossed (Holowenko and Fedorak 2001). Percentage of ions observed through MS would be entered into cells corresponding to naphthenic acids. Figure 1.5, taken from Holowenko and Fedorak (2001) is an example of a three-dimensional graph generated from Table 1.6. The Suncor tailings pond sample has a bimodal distribution. A valley is observed from carbons 19 through 23 and a higher relative abundance of ions is found on either side of this valley (Holowenko and Fedorak 2001, Holowenko et al. 2002). The group of compounds with greater than 22 carbons, as seen in Figure 1.5, is known as the C22+ cluster. This representation may help determine which naphthenic acid components are more problematic, or more easily biodegraded (Holowenko and Fedorak 2001, Holowenko et al. 2002). As promising and useful as the discussed MS methods are in determining naphthenic acids composition, they have their limits. Commonly used MS can detect ions at low and high resolution. These describe the precision of the instrument in determining the mass of the ion. Figure 1.6 shows four compounds with carboxylic acid moieties.

Nominal and accurate masses quoted here were calculated using the program available from Manura and Manura (2002). All four compounds have a nominal weight of 248 Da. Compound "a" contains one less carbon, four less hydrogens, and one more oxygen compared to compounds "b", "c", and "d". Despite that, low resolution MS cannot differentiate among the four compounds. Notice that compounds "a" and "d" do not correspond to naphthenic acids. They will nevertheless be considered as naphthenic acids, like compounds "b" and "c", with 16 carbons, and four double bond equivalents (Z=-8) (Table 1.5). High resolution MS, on the other hand, could differentiate the non-naphthenic acid compound "a", from compounds "b", "c", and "d". Compound "a" has a formula weight of 248.14126 Da, while "b", "c" and "d" have formula weights of 248.177639 Da. The 0.0364 difference in masses could be resolved by high resolution

Table 1.5 Expected nominal weight as observed by MS (underivatized), based on carbon number (n) and Z family combinations, which fulfill the "naphthenic acid" definition, given the formula $C_nH_{2n+Z}O_2$. Cells with a diagonal line represent theoretical compounds not considered as naphthenic acids.

Carbon number	Z Family						
	0	-2	-4	-6	-8	-10	-12
5	$C_5H_{10}O_2$[a] 102[b]	$C_5H_8O_2$ 100	$C_5H_6O_2$ 98	$C_5H_4O_2$ 96	$C_5H_2O_2$ 94	C_5O_2 92	---
6	$C_6H_{12}O_2$ 116	$C_6H_{10}O_2$ 114	$C_6H_8O_2$ 112	$C_6H_6O_2$ 110	$C_6H_4O_2$ 108	$C_6H_2O_2$ 106	C_6O_2 104
7	$C_7H_{14}O_2$ 130	$C_7H_{12}O_2$ 128	$C_7H_{10}O_2$ 126	$C_7H_8O_2$ 124	$C_7H_6O_2$ 122	$C_7H_4O_2$ 120	$C_7H_2O_2$ 118
8	$C_8H_{16}O_2$ 144	$C_8H_{14}O_2$ 1 42	$C_8H_{12}O_2$ 140	$C_8H_{10}O_2$ 138	$C_8H_8O_2$ 136	$C_8H_6O_2$ 134	$C_8H_4O_2$ 132
9	$C_9H_{18}O_2$ 158	$C_9H_{16}O_2$ 156	$C_9H_{14}O_2$ 154	$C_9H_{12}O_2$ 152	$C_9H_{10}O_2$ 150	$C_9H_8O_2$ 148	$C_9H_6O_2$ 146
10	$C_{10}H_{20}O_2$ 172	$C_{10}H_{18}O_2$ 170	$C_{10}H_{16}O_2$ 168	$C_{10}H_{14}O_2$ 166	$C_{10}H_{12}O_2$ 164	$C_{10}H_{10}O_2$ 162	$C_{10}H_8O_2$ 160
11	$C_{11}H_{22}O_2$ 186	$C_{11}H_{20}O_2$ 184	$C_{11}H_{18}O_2$ 182	$C_{11}H_{16}O_2$ 180	$C_{11}H_{14}O_2$ 178	$C_{11}H_{12}O_2$ 176	$C_{11}H_{10}O_2$ 174
12	$C_{12}H_{24}O_2$ 200	$C_{12}H_{22}O_2$ 198	$C_{12}H_{20}O_2$ 196	$C_{12}H_{18}O_2$ 194	$C_{12}H_{16}O_2$ 192	$C_{12}H_{14}O_2$ 190	$C_{12}H_{12}O_2$ 188
13	$C_{13}H_{26}O_2$ 214	$C_{13}H_{24}O_2$ 212	$C_{13}H_{22}O_2$ 210	$C_{13}H_{20}O_2$ 208	$C_{13}H_{18}O_2$ 206	$C_{13}H_{16}O_2$ 204	$C_{13}H_{14}O_2$ 202
14	$C_{14}H_{28}O_2$ 228	$C_{14}H_{26}O_2$ 226	$C_{14}H_{24}O_2$ 224	$C_{14}H_{22}O_2$ 222	$C_{14}H_{20}O_2$ 220	$C_{14}H_{18}O_2$ 218	$C_{14}H_{16}O_2$ 216
15	$C_{15}H_{30}O_2$ 242	$C_{15}H_{28}O_2$ 240	$C_{15}H_{26}O_2$ 238	$C_{15}H_{24}O_2$ 236	$C_{15}H_{22}O_2$ 234	$C_{15}H_{20}O_2$ 232	$C_{15}H_{18}O_2$ 230
16	$C_{16}H_{32}O_2$ 256	$C_{16}H_{30}O_2$ 254	$C_{16}H_{28}O_2$ 252	$C_{16}H_{26}O_2$ 250	$C_{16}H_{24}O_2$ 248	$C_{16}H_{22}O_2$ 246	$C_{16}H_{20}O_2$ 244
17	$C_{17}H_{34}O_2$ 270	$C_{17}H_{32}O_2$ 268	$C_{17}H_{30}O_2$ 266	$C_{17}H_{28}O_2$ 264	$C_{17}H_{26}O_2$ 262	$C_{17}H_{24}O_2$ 260	$C_{17}H_{22}O_2$ 258
18	$C_{18}H_{36}O_2$ 284	$C_{18}H_{34}O_2$ 282	$C_{18}H_{32}O_2$ 280	$C_{18}H_{30}O_2$ 278	$C_{18}H_{28}O_2$ 276	$C_{18}H_{26}O_2$ 274	$C_{18}H_{24}O_2$ 272
19	$C_{19}H_{38}O_2$ 298	$C_{19}H_{36}O_2$ 296	$C_{19}H_{34}O_2$ 294	$C_{19}H_{32}O_2$ 292	$C_{19}H_{30}O_2$ 290	$C_{19}H_{28}O_2$ 288	$C_{19}H_{26}O_2$ 286
20	$C_{20}H_{40}O_2$ 312	$C_{20}H_{38}O_2$ 310	$C_{20}H_{36}O_2$ 308	$C_{20}H_{34}O_2$ 306	$C_{20}H_{32}O_2$ 304	$C_{20}H_{30}O_2$ 302	$C_{20}H_{28}O_2$ 300
21	$C_{21}H_{42}O_2$ 326	$C_{21}H_{40}O_2$ 324	$C_{21}H_{38}O_2$ 322	$C_{21}H_{36}O_2$ 320	$C_{21}H_{34}O_2$ 318	$C_{21}H_{32}O_2$ 316	$C_{21}H_{30}O_2$ 314
22	$C_{22}H_{44}O_2$ 340	$C_{22}H_{42}O_2$ 338	$C_{22}H_{40}O_2$ 336	$C_{22}H_{38}O_2$ 334	$C_{22}H_{36}O_2$ 332	$C_{22}H_{34}O_2$ 330	$C_{22}H_{32}O_2$ 328
23	$C_{23}H_{46}O_2$ 354	$C_{23}H_{44}O_2$ 352	$C_{23}H_{42}O_2$ 350	$C_{23}H_{40}O_2$ 348	$C_{23}H_{38}O_2$ 346	$C_{23}H_{36}O_2$ 344	$C_{23}H_{34}O_2$ 342
24	$C_{24}H_{48}O_2$ 368	$C_{24}H_{46}O_2$ 366	$C_{24}H_{44}O_2$ 364	$C_{24}H_{42}O_2$ 362	$C_{24}H_{40}O_2$ 360	$C_{24}H_{38}O_2$ 358	$C_{24}H_{36}O_2$ 356
25	$C_{25}H_{50}O_2$ 382	$C_{25}H_{48}O_2$ 380	$C_{25}H_{46}O_2$ 378	$C_{25}H_{44}O_2$ 376	$C_{25}H_{42}O_2$ 374	$C_{25}H_{40}O_2$ 372	$C_{25}H_{38}O_2$ 370
26	$C_{26}H_{52}O_2$ 396	$C_{26}H_{50}O_2$ 394	$C_{26}H_{48}O_2$ 392	$C_{26}H_{46}O_2$ 390	$C_{26}H_{44}O_2$ 388	$C_{26}H_{42}O_2$ 386	$C_{26}H_{40}O_2$ 384
27	$C_{27}H_{54}O_2$ 410	$C_{27}H_{52}O_2$ 408	$C_{27}H_{50}O_2$ 406	$C_{27}H_{48}O_2$ 404	$C_{27}H_{46}O_2$ 402	$C_{27}H_{44}O_2$ 400	$C_{27}H_{42}O_2$ 398
28	$C_{28}H_{56}O_2$ 424	$C_{28}H_{54}O_2$ 422	$C_{28}H_{52}O_2$ 420	$C_{28}H_{50}O_2$ 418	$C_{28}H_{48}O_2$ 416	$C_{28}H_{46}O_2$ 414	$C_{28}H_{44}O_2$ 412
29	$C_{29}H_{58}O_2$ 438	$C_{29}H_{56}O_2$ 436	$C_{29}H_{54}O_2$ 434	$C_{29}H_{52}O_2$ 432	$C_{29}H_{50}O_2$ 430	$C_{29}H_{48}O_2$ 428	$C_{29}H_{46}O_2$ 426
30	$C_{30}H_{60}O_2$ 452	$C_{30}H_{58}O_2$ 450	$C_{30}H_{56}O_2$ 448	$C_{30}H_{54}O_2$ 446	$C_{30}H_{52}O_2$ 444	$C_{30}H_{50}O_2$ 442	$C_{30}H_{48}O_2$ 440
31	$C_{31}H_{62}O_2$ 466	$C_{31}H_{60}O_2$ 464	$C_{31}H_{58}O_2$ 462	$C_{31}H_{56}O_2$ 460	$C_{31}H_{54}O_2$ 458	$C_{31}H_{52}O_2$ 456	$C_{31}H_{50}O_2$ 454
32	$C_{32}H_{64}O_2$ 480	$C_{32}H_{62}O_2$ 478	$C_{32}H_{60}O_2$ 476	$C_{32}H_{58}O_2$ 474	$C_{32}H_{56}O_2$ 472	$C_{32}H_{54}O_2$ 470	$C_{32}H_{52}O_2$ 468
33	$C_{33}H_{66}O_2$ 494	$C_{33}H_{64}O_2$ 492	$C_{33}H_{62}O_2$ 490	$C_{33}H_{60}O_2$ 488	$C_{33}H_{58}O_2$ 486	$C_{33}H_{56}O_2$ 484	$C_{33}H_{54}O_2$ 482

[a] Chemical formula corresponding to the expected mass

[b] Expected formula weight for a compound with the corresponding carbon number and Z family

Table 1.6 Expected nominal weight of the *t*-butyldimethylsilyl ester, $[M+57]^+$ion based on carbon number (n) and Z family combinations, which fulfill the "naphthenic acid" definition, given the formula $C_nH_{2n+Z}O_2$. Cells with a diagonal line represent theoretical compounds not considered as naphthenic acids.

Carbon number	Z Family						
	0	-2	-4	-6	-8	-10	-12
5	$C_5H_{10}O_2$[a] 159[b]	$C_5H_8O_2$ 157	$C_5H_6O_2$ 155	$C_5H_4O_2$ 153	$C_5H_2O_2$ 151	C_5O_2 149	---
6	$C_6H_{12}O_2$ 173	$C_6H_{10}O_2$ 171	$C_6H_8O_2$ 170	$C_6H_6O_2$ 168	$C_6H_4O_2$ 166	$C_6H_2O_2$ 164	C_6O_2 162
7	$C_7H_{14}O_2$ 187	$C_7H_{12}O_2$ 185	$C_7H_{10}O_2$ 183	$C_7H_8O_2$ 181	$C_7H_6O_2$ 179	$C_7H_4O_2$ 177	$C_7H_2O_2$ 175
8	$C_8H_{16}O_2$ 201	$C_8H_{14}O_2$ 199	$C_8H_{12}O_2$ 197	$C_8H_{10}O_2$ 195	$C_8H_8O_2$ 193	$C_8H_6O_2$ 191	$C_8H_4O_2$ 189
9	$C_9H_{18}O_2$ 215	$C_9H_{16}O_2$ 213	$C_9H_{14}O_2$ 211	$C_9H_{12}O_2$ 209	$C_9H_{10}O_2$ 207	$C_9H_8O_2$ 205	$C_9H_6O_2$ 203
10	$C_{10}H_{20}O_2$ 229	$C_{10}H_{18}O_2$ 227	$C_{10}H_{16}O_2$ 225	$C_{10}H_{14}O_2$ 223	$C_{10}H_{12}O_2$ 221	$C_{10}H_{10}O_2$ 219	$C_{10}H_8O_2$ 217
11	$C_{11}H_{22}O_2$ 243	$C_{11}H_{20}O_2$ 241	$C_{11}H_{18}O_2$ 239	$C_{11}H_{16}O_2$ 237	$C_{11}H_{14}O_2$ 235	$C_{11}H_{12}O_2$ 233	$C_{11}H_{10}O_2$ 231
12	$C_{12}H_{24}O_2$ 257	$C_{12}H_{22}O_2$ 255	$C_{12}H_{20}O_2$ 253	$C_{12}H_{18}O_2$ 251	$C_{12}H_{16}O_2$ 249	$C_{12}H_{14}O_2$ 247	$C_{12}H_{12}O_2$ 245
13	$C_{13}H_{26}O_2$ 271	$C_{13}H_{24}O_2$ 269	$C_{13}H_{22}O_2$ 267	$C_{13}H_{20}O_2$ 265	$C_{13}H_{18}O_2$ 263	$C_{13}H_{16}O_2$ 261	$C_{13}H_{14}O_2$ 259
14	$C_{14}H_{28}O_2$ 285	$C_{14}H_{26}O_2$ 283	$C_{14}H_{24}O_2$ 281	$C_{14}H_{22}O_2$ 279	$C_{14}H_{20}O_2$ 277	$C_{14}H_{18}O_2$ 275	$C_{14}H_{16}O_2$ 273
15	$C_{15}H_{30}O_2$ 299	$C_{15}H_{28}O_2$ 297	$C_{15}H_{26}O_2$ 295	$C_{15}H_{24}O_2$ 293	$C_{15}H_{22}O_2$ 291	$C_{15}H_{20}O_2$ 289	$C_{15}H_{18}O_2$ 287
16	$C_{16}H_{32}O_2$ 313	$C_{16}H_{30}O_2$ 311	$C_{16}H_{28}O_2$ 309	$C_{16}H_{26}O_2$ 307	$C_{16}H_{24}O_2$ 305	$C_{16}H_{22}O_2$ 303	$C_{16}H_{20}O_2$ 301
17	$C_{17}H_{34}O_2$ 327	$C_{17}H_{32}O_2$ 325	$C_{17}H_{30}O_2$ 323	$C_{17}H_{28}O_2$ 321	$C_{17}H_{26}O_2$ 319	$C_{17}H_{24}O_2$ 317	$C_{17}H_{22}O_2$ 315
18	$C_{18}H_{36}O_2$ 341	$C_{18}H_{34}O_2$ 339	$C_{18}H_{32}O_2$ 337	$C_{18}H_{30}O_2$ 335	$C_{18}H_{28}O_2$ 333	$C_{18}H_{26}O_2$ 331	$C_{18}H_{24}O_2$ 329
19	$C_{19}H_{38}O_2$ 355	$C_{19}H_{36}O_2$ 353	$C_{19}H_{34}O_2$ 351	$C_{19}H_{32}O_2$ 349	$C_{19}H_{30}O_2$ 347	$C_{19}H_{28}O_2$ 345	$C_{19}H_{26}O_2$ 343
20	$C_{20}H_{40}O_2$ 369	$C_{20}H_{38}O_2$ 367	$C_{20}H_{36}O_2$ 365	$C_{20}H_{34}O_2$ 363	$C_{20}H_{32}O_2$ 361	$C_{20}H_{30}O_2$ 359	$C_{20}H_{28}O_2$ 357
21	$C_{21}H_{42}O_2$ 383	$C_{21}H_{40}O_2$ 381	$C_{21}H_{38}O_2$ 379	$C_{21}H_{36}O_2$ 377	$C_{21}H_{34}O_2$ 375	$C_{21}H_{32}O_2$ 373	$C_{21}H_{30}O_2$ 371
22	$C_{22}H_{44}O_2$ 397	$C_{22}H_{42}O_2$ 395	$C_{22}H_{40}O_2$ 393	$C_{22}H_{38}O_2$ 391	$C_{22}H_{36}O_2$ 389	$C_{22}H_{34}O_2$ 387	$C_{22}H_{32}O_2$ 385
23	$C_{23}H_{46}O_2$ 411	$C_{23}H_{44}O_2$ 409	$C_{23}H_{42}O_2$ 407	$C_{23}H_{40}O_2$ 405	$C_{23}H_{38}O_2$ 403	$C_{23}H_{36}O_2$ 401	$C_{23}H_{34}O_2$ 399
24	$C_{24}H_{48}O_2$ 425	$C_{24}H_{46}O_2$ 423	$C_{24}H_{44}O_2$ 421	$C_{24}H_{42}O_2$ 419	$C_{24}H_{40}O_2$ 417	$C_{24}H_{38}O_2$ 415	$C_{24}H_{36}O_2$ 413
25	$C_{25}H_{50}O_2$ 439	$C_{25}H_{48}O_2$ 437	$C_{25}H_{46}O_2$ 435	$C_{25}H_{44}O_2$ 433	$C_{25}H_{42}O_2$ 431	$C_{25}H_{40}O_2$ 429	$C_{25}H_{38}O_2$ 427
26	$C_{26}H_{52}O_2$ 453	$C_{26}H_{50}O_2$ 451	$C_{26}H_{48}O_2$ 449	$C_{26}H_{46}O_2$ 447	$C_{26}H_{44}O_2$ 445	$C_{26}H_{42}O_2$ 443	$C_{26}H_{40}O_2$ 441
27	$C_{27}H_{54}O_2$ 467	$C_{27}H_{52}O_2$ 465	$C_{27}H_{50}O_2$ 463	$C_{27}H_{48}O_2$ 461	$C_{27}H_{46}O_2$ 459	$C_{27}H_{44}O_2$ 457	$C_{27}H_{42}O_2$ 455
28	$C_{28}H_{56}O_2$ 481	$C_{28}H_{54}O_2$ 479	$C_{28}H_{52}O_2$ 477	$C_{28}H_{50}O_2$ 475	$C_{28}H_{48}O_2$ 473	$C_{28}H_{46}O_2$ 471	$C_{28}H_{44}O_2$ 469
29	$C_{29}H_{58}O_2$ 495	$C_{29}H_{56}O_2$ 493	$C_{29}H_{54}O_2$ 491	$C_{29}H_{52}O_2$ 489	$C_{29}H_{50}O_2$ 487	$C_{29}H_{48}O_2$ 485	$C_{29}H_{46}O_2$ 483
30	$C_{30}H_{60}O_2$ 509	$C_{30}H_{58}O_2$ 507	$C_{30}H_{56}O_2$ 505	$C_{30}H_{54}O_2$ 503	$C_{30}H_{52}O_2$ 501	$C_{30}H_{50}O_2$ 499	$C_{30}H_{48}O_2$ 497
31	$C_{31}H_{62}O_2$ 523	$C_{31}H_{60}O_2$ 521	$C_{31}H_{58}O_2$ 519	$C_{31}H_{56}O_2$ 517	$C_{31}H_{54}O_2$ 515	$C_{31}H_{52}O_2$ 513	$C_{31}H_{50}O_2$ 511
32	$C_{32}H_{64}O_2$ 537	$C_{32}H_{62}O_2$ 535	$C_{32}H_{60}O_2$ 533	$C_{32}H_{58}O_2$ 531	$C_{32}H_{56}O_2$ 529	$C_{32}H_{54}O_2$ 527	$C_{32}H_{52}O_2$ 525
33	$C_{33}H_{66}O_2$ 551	$C_{33}H_{64}O_2$ 549	$C_{33}H_{62}O_2$ 547	$C_{33}H_{60}O_2$ 545	$C_{33}H_{58}O_2$ 543	$C_{33}H_{56}O_2$ 541	$C_{33}H_{54}O_2$ 539

[a] Chemical formula corresponding to the expected mass

[b] Expected formula weight for a compound with the corresponding carbon number and Z family

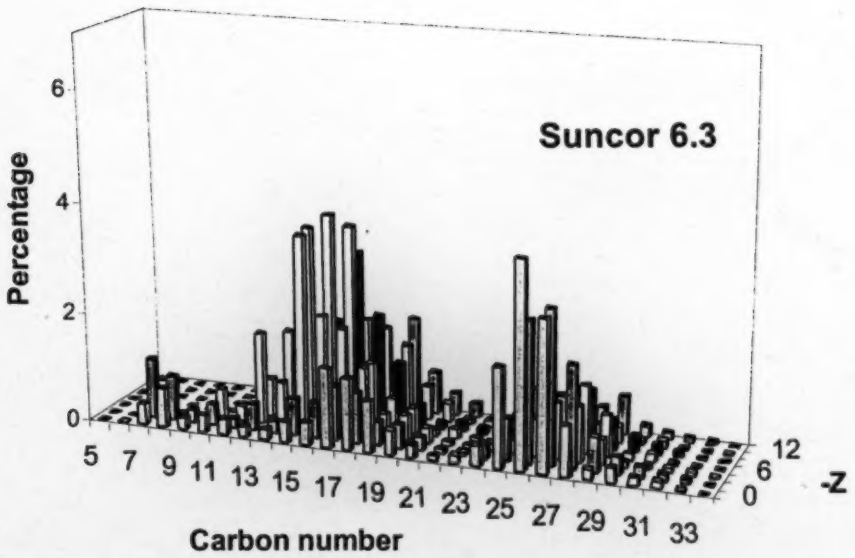
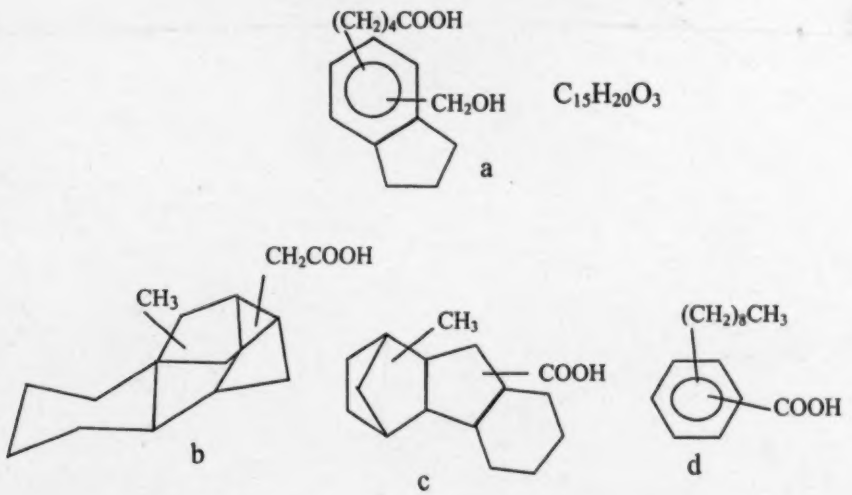


Figure 1.5 Three-dimensional representation of naphthenic acids dimethylsilyl esters from tailings pond water (Holowenko and Fedorak 2001).



$C_{16}H_{24}O_2$

$M^+ = 248$

Figure 1.6 Compounds considered naphthenic acids by low or high resolution MS based on the detected mass. All compounds have a nominal m/z of 248 Da.

MS. Unfortunately, not even high resolution MS could distinguish among compounds "b", "c" and "d" in a complex mixture such as naphthenic acids. MS on its own cannot specify the exact structure of a naphthenic acid compound in a complex mixture (Hsu et al. 2000, Qian et al. 2001, Rudzinski et al. 2002, and Barrow et al. 2003).

Derivatizing the naphthenic acids for MS presents another complication (Figure 1.7). St. John et al. (1998) have demonstrated that both carboxylic acid groups in a dicarboxylic acid are derivatized by MTBSTFA, but only one *t*-butyldimethylsilyl is ionized. Figure 1.7A represents a derivatized naphthenic acid. The dicarboxylic acid presented by Figure 1.7B would be considered to be a naphthenic acid whether or not it was derivatized (Tables 1.5, 1.6). Underivatized, it has a formula weight of 158 Da, which corresponds to an acyclic compound with nine carbons ($C_9H_{18}O_2$) (Table 1.5). After derivatization, using the method developed by St. John et al. (1998), Holowenko and Fedorak (2001), and Holowenko et al. (2002), the compound would be determined to have the molecular formula $C_{18}H_{24}O_2$ (Table 1.6). In addition to that, if it had two more double bonds, the underivatized compound would not be considered a naphthenic acid ($M^+ = 154$, Table 1.5), but the derivatized compound would be considered a naphthenic acid with the formula $C_{17}H_{32}O_2$ ($[M+57]^+ = 325$, Table 1.6). Luckily, commercial naphthenic acids do not contain dicarboxylic acids and alkynes (St. John et al. 1998).

1.6.5 Other MS methods, in combination with IR and nuclear magnetic resonance spectroscopy

As demonstrated in the preceding section, it is difficult to determine the composition of naphthenic acids from MS results alone (Hsu et al. 2000, Qian et al. 2001, Rudzinski et al. 2002, Barrow et al. 2003). Early studies (from the 1960s and 1970s) have shown that the acidic fraction of petroleum consists of branched alkane chains, which are mainly monobasic (Lebedevskaya et al. 1978). UV and IR have identified pyrroles, thiophenes, phenols, and possibly benzofurans in naphthenic acids purified from California crude oil (Seifert et al. 1969). Thin layer chromatography of California crude demonstrated that the naphthenic acid fraction contains 3 mol% phenol, 5 mol% nitrogen, (2% of which are in indoles), and 8.5 mol% sulfur (Seifert and Teeter 1969). Such

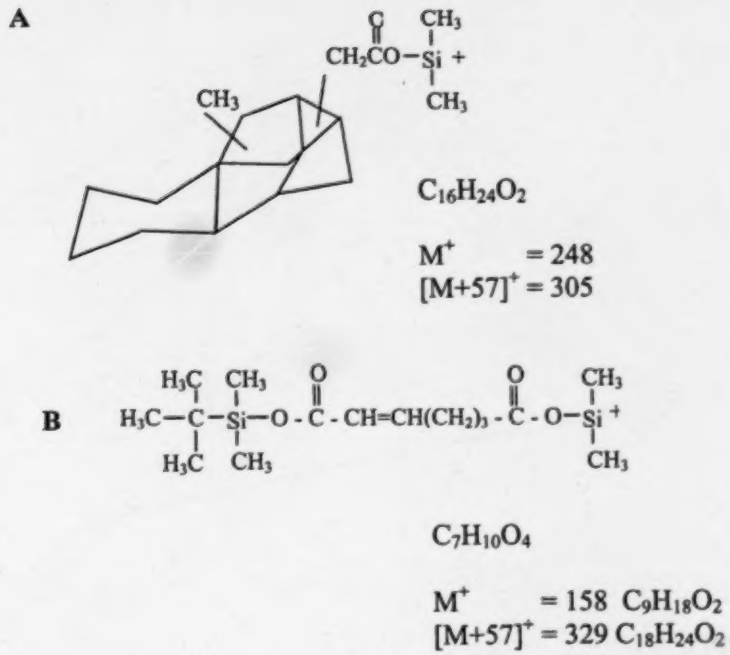


Figure 1.7 Effect of derivatization on the mass observed by MS for naphthenic acid (A) and non-naphthenic acid compounds (B).

findings challenge the established definition of naphthenic acids.

More recent experiments have provided further proof that crude oil fractions, which are believed to contain only naphthenic acids, may contain other compounds as well. FT-IR, along with high resolution MS have been used to characterize San Joaquin valley, California crude oil (Tomczyk and Winans 2001). FT-IR determined the presence of aromatics, in agreement with high resolution MS. Possible combinations of atoms were calculated to determine the identity of ions found using high resolution MS. They found that 40% of the acids did not contain the carboxylic acid functional group. These compounds contain heteroatoms such as sulfur and nitrogen. In addition to that, 85% of the carboxylic acid compounds contain other heteroatoms, besides the two oxygens found in naphthenic acids. O_4, S, N_2, NO, NO_2, N_2O, N_2O_2 (these do not necessarily represent functional groups) were also identified. Aromatics make up 3.1% of California crude oil acids as demonstrated by MS (Hsu et al. 2000). These results are similar to those they

determined using nuclear magnetic resonance, where aromatics were found to make up 5.4% of the crude oil acids.

Tandem MS determines the presence of functional groups by measuring the difference in mass between the parent and daughter ions. The daughter ions are not always detected by MS, because they are at times, neutral. This procedure has been used by Rudzinski et al. (2002) to characterize acids from Maya crude, as well as commercial naphthenic acids from Fluka, and Pfaltz and Bauer. Parent ions from the commercial preparations lost only CO_2 and H_2O in producing daughter ions. Parent ions from the Maya crude oil extract on the other hand lost SO_2, SO_3^-, and HSO_3^-, in addition to CO_2. Unlike the commercial preparations therefore, the Maya crude oil extract mainly consists of alkylsulfonic acids.

Ion cyclotron resonance has increased the resolution of MS, defined as ultrahigh resolution MS. Barrow et al. (2003) found that nominal ions, particularly in compounds with more negative Z numbers, consist of doublets. South American heavy crude was analyzed by Qian et al. (2001) using ion cyclotron resonance. Naphthenic acids make up less than half of the acidic extract. They found aromatic, sulfur, and polyaromatic containing compounds. Results similar to those reported by Rudzinski et al. (2002) were also observed in that SO_3, and SO_2 containing compounds were observed. They found compounds with O_4, similar to Tomczyk et al. (2001), in addition to O_3-containing compounds.

FT-IR and tandem MS may be capable of differentiating compound "d" from "b", and "c" (Figure 1.6). This is believed to be possible by comparing the daughter ions of samples and known standards (Barrow et al. 2003). IR absorbance by aromatic compounds in a naphthenic acids mixture has been demonstrated by Seifert and Teeter (1969), Seifert et al. (1969), and Tomczyk and Winans (2001). Nuclear magnetic resonance can also detect the presence of aromatics (Hsu et al. 2000), though with less sensitivity compared to FT-IR (Tomczyk and Winans 2001).

1.6.6 Analysis of Athabasca oil sands tailings ponds water using MS

Naphthenic acids in tailings water are a primary concern because of their toxicity.

MS has been used to characterize the composition of naphthenic acids in these waters (Morales et al. 1993, Morales-Izquierdo 1999, Holowenko et al. 2002, Rogers et al. 2002a, Gabryelski and Froese 2003, Lo et al. 2003). Tailings water sources include Mildred Lake Settling Basin, which contained 49 mg naphthenic acids/L, and Suncor consolidated tailings release water, which had 68 mg naphthenic acids/L (Holowenko et al. 2002).

The dimethylsilyl ester ion distribution of an oil sands ore and a Mildred Lake Settling Basin extract (MLSBF) were found to be similar to each other. Holowenko et al. (2002) found that the majority of ions from the oil sands ore had 14 to 18 carbons, mainly with two and three rings. MLSBF naphthenic acids mainly had 13 to 16 carbons, with one, two, and three rings (two and three ring compounds predominate). Both samples had few ions in the C22+ cluster. A section of the Suncor consolidated tailings release water naphthenic acids envelope was similar to that of MLSBF. The most abundant ions had 13 to 16 carbons, in addition to compounds with two and three rings, four ring compounds were also relatively abundant. The Suncor sample also had a higher percentage of compounds with 22 carbons or more.

Other investigators have analyzed the Mildred Lake Settling Basin tailings water naphthenic acids component. Morales et al. (1993) used negative fast atom bombardment-MS, using a triethanolamine matrix. They found that compounds in the Z=-4 series (two rings) were relatively more abundant, followed by the Z=-6 (three rings) series, whereas those in the Z=0 series were sparse. Analysis using electrospray ionization-MS was also performed by Lo et al. (2003). Their data support those of Holowenko et al. (2002) and Morales et al. (1993) in that the Z=-4 series was found to be the most abundant. As found by Holowenko et al. (2002), Lo et al. (2003) found that the majority of the ions (60%) have 13 to 19 carbons. However, there were also several differences in the distribution observed by Lo et al. (2003). They found a higher proportion of the C22+ cluster, compared to the data of Holowenko et al. (2002). The presence of compounds with aromatic rings and double bonds was also postulated by Lo et al. (2003) because of ions with 15 to 18 carbons in the Z=-8 to Z=-12 families, which would not fit the naphthenic acid definition.

Morales-Izquierdo (1999) also analyzed Mildred Lake Settling Basin tailings water using electrospray ionization-MS. It was found that the distributions across Z values were similar. Rogers et al. (2002a) found results similar to those of Morales-Izquierdo (1999) after electrospray ionization-MS analysis. The relative abundances across Z families from Z=0 to Z=-8 were similar at ~20%. The naphthenic acids analysis by Rogers et al. (2002a) were restricted to those with 14 to 24 carbons. As found by Lo et al. (2003), carbon numbers 17 to 19 dominated the mixture.

A novel method using high-field asymmetric waveform ion mobility spectrometry MS, coupled to electrospray ionization and mass filters such as time of flight and quadrupole, was used by Gabryelski and Froese (2003) to analyze naphthenic acids. They found several differences between naphthenic acids from ores and those from tailings waters. Naphthenic acids from tailings waters have a greater proportion of a 253 Da ion transmitted through the high-field asymmetric waveform ion mobility analyzer. This ion was believed to result from the biodegradation of a 373 Da ion, which along with the 417 Da, and 461 Da ions were found only in the ore samples.

1.7 Naphthenic acids and surrogate naphthenic acids biodegradation

1.7.1 Surrogate naphthenic acids

The complexity of naphthenic acids mixtures makes it difficult to determine the effects of structure on biodegradation. Catabolism of several compounds which follow the general naphthenic acid formula $C_nH_{2n+Z}O_2$ have been studied (Table 1.7) in an attempt to characterize the effects of structure on the extent of biodegradation. These compounds represent substitution patterns found in naphthenic acid mixtures. Cyclohexanecarboxylic acid, cyclohexanepropanoic acid, cyclohexanebutanoic acid, and cyclohexanepentanoic acid are cycloalkanes substituted with an alkanoic acid. Cycloalkanes with both an alkyl and a carboxylic acid substitution include 2-methyl-cyclohexanecarboxylic acid, and *trans*-4-pentylcyclohexanecarboxylic acid. Decahydro-2-naphthoic acid is a bicyclic cycloalkane with a carboxylic acid substituent.

Studies using the compounds listed in Table 1.7 have shown that different

Table 1.7 Surrogate naphthenic acids used to characterize naphthenic acids biodegradation.

Compound	Reference	Organism or culture used
Cyclohexanecarboxylic acid	Blakley 1974	*Arthrobacter* sp.
	Kaneda 1974	*Corynebacterium cyclohexanicum*
	Rho and Evans 1975	*Acinetobacter anitratum*
	Smith and Callely 1975	*Arthrobacter* sp.
	Blakley 1978	*Alcaligenes faecalis*
	Taylor and Trudgill 1978	*Alcaligenes* strain W1
	Blakley and Papish 1982	*Pseudomonas putida*
	Herman et al. 1994	Oil sands tailings aerobic microcosms
	Kuver et al. 1995	*Rhodopseudomonas palustris*
	Dutta and Harayama 2001	*Alcanivorax* sp.
Cyclohexanepropanoic acid	Holowenko et al. 2001	Methanogenic microcosm from Base mine tailings, and sewage sludge
Cyclohexanebutanoic acid	Holowenko et al. 2001	Methanogenic microcosm from sewage sludge
Cyclohexanepentanoic acid	Holowenko et al. 2001	Methanogenic microcosm from Base mine tailings
	Herman et al. 1994	Oil sands tailings aerobic microcosms
2-Methylcyclohexane carboxylic acid	Herman et al. 1994	Oil sands tailings aerobic microcosms
trans-4-Pentyl-cyclohexanecarboxylic acid	Herman et al. 1994	Oil sands tailings aerobic microcosms
Decahydro-2-naphthoic acid	Lai et al. 1996	Oil sands tailings aerobic microcosms

substitutions are related to the recalcitrance of the carboxylic acid. Holowenko et al. (2001) studied methanogenic microcosms with bacteria from sewage sludge and tailings waters. Using sewage sludge samples as the inoculum source, it was determined that only the alkane substituent of cyclohexanepentanoic acid was metabolized to methane.

For cyclohexanepropanoic acid, however, both the side chain and ring were metabolized. Studies using ^{14}C-labeled decahydro-2-naphthoic acid showed that this compound was not utilized by methanogenic microcosms. Compound structure, including the alkanoic acid moiety attached to the cycloalkane, affects the biodegradability of the compounds. This may help explain why Holowenko et al. (2001) found that commercial and tailings extract naphthenic acids do not stimulate methanogenesis.

Cyclohexanes substituted with both an alkyl and an alkanoic acid moiety were less susceptible to aerobic biodegradation compared to cyclohexanes substituted with only an alkanoic acid moiety (Herman et al. 1994). Bacterial communities from tailings pond water, acclimated on either a commercial naphthenic acids mixture, or a tailings pond extract as well as bacteria from tailings pond water (unacclimated), were incubated with one of the surrogate acids for 24 days. About 50% of cyclohexanecarboxylic acid carbon and cyclohexanepentanoic acid carbon were mineralized to CO_2 by the three communities. About 50% of methylcyclohexanecarboxylic acid carbon was also mineralized to CO_2 by the community acclimated on commercial naphthenic acids, and unacclimated tailings pond water. However, only 7% of the carbon from this compound was mineralized to CO_2 by the community acclimated on tailings extract. This is comparable to the mineralization of 6% pentylcyclohexanecarboxylic acid carbon to CO_2 by the community acclimated on commercial naphthenic acids. The community acclimated on tailings pond extract, as well as the unacclimated community both mineralized 24% of the carbon from this compound to CO_2. The lowered CO_2 production indicates that alkyl substituted compounds are less readily degraded.

Experiments using ^{14}C-labeled decahydro-2-naphthoic acid showed that fused rings are susceptible to aerobic mineralization by communities in tailings pond water (Lai et al. 1996). Two to four percent of this compound was transformed to CO_2 after 8 weeks (controls generated a background CO_2 level of 0.5%). This biodegradation rate was much slower than the 15% CO_2 production from palmitic acid after 4 weeks. These values cannot be compared to those observed by Herman et al. (1994) because of differences in the activity of bacterial communities used. To clarify, palmitic acid can be taken as the normalizing compound. Whereas Lai et al. (1996) observed only 15% of the palmitic acid carbon transformed to CO_2 after 28 days, Herman et al. (1994) observed

that 37% to 64% of the palmitic acid carbon was transformed to CO_2 after 24 days. Nevertheless, this demonstrates that tailings pond bacteria can mineralize fused rings.

Anaerobic biodegradation of acyclic carboxylic acids with tertiary and quaternary carbons were studied by Chua et al. (2001). They found that compounds with tertiary compounds at both the α (2-ethylhexanoic acid, 2-methylhexanoic acid) and β (3-ethylhexanoic acid, and 3-methylhexanoic acid) positions were biodegraded by a mixed anaerobic community composed of *Syntrophomonas* spp. *Methanothrix* spp. *Methanosarcina* spp., and *Methanococcus* spp. The degradation rates for the compounds containing tertiary carbons ranged from 5.0 to 8.5 µmol/h. The bacterial consortium did not metabolize compounds containing a quaternary carbon.

Cyclohexanecarboxylic acid biodegradation is the most studied thus far (Table 1.7). It has been chosen as the model naphthenic acid to help elucidate possible biodegradation pathways involved in cycloalkyl carboxylic acid biodegradation (Blakley 1974, Kaneda 1974, Rho and Evans 1975, Smith and Callely 1975, Blakley 1978, Taylor and Trudgill 1978, Blakley and Papish 1982, Kuver et al.1995, Dutta and Harayama 2001).

1.7.2 Possible aerobic biodegradation pathways

Figure 1.8 presents a synthesis of the intermediates identified, together with the pathways and steps proposed to be involved in cyclohexanecarboxylic acid catabolism (Blakley 1974, Kaneda 1974, Rho and Evans 1975, Smith and Callely 1975, Blakley 1978, Taylor and Trudgill 1978, Blakley and Papish 1982, Dutta and Harayama 2001). There are two major pathways involved in cyclohexanecarboxylic acid metabolism. The first occurs by alicyclic ring cleavage followed by β-oxidation (Rho and Evans 1975, Blakley 1978, Blakley and Papish 1982) (Figure 1.8, path 1). The second occurs by cyclohexanecarboxylic acid aromatization followed by ring fission (Kaneda 1974, Blakley 1974, Smith and Callely 1975, Taylor and Trudgill 1978) (Figure 1.8, paths 3 and 4).

Evidence for cleavage of the alicyclic ring followed by β-oxidation was first observed by Rho and Evans (1975) using *Acinetobacter anitratum*. Cyclohexane

carboxylate labeled with ^{14}C (carboxyl group) was used to help track the intermediates. Pimelic acid (Figure 1.8, compound e) was isolated and identified by comparing the elution of the unidentified compound with known standards on a thin layer chromatography plate. Metabolites such as cyclohex-1-enecarboxylic acid (Figure 1.8, compound b), 2-hydroxycyclohexanecarboxylic acid (Figure 1.8, compound c), and pimelic acid were also identified using MS of their methyl esters. Metabolic and enzymatic experiments were performed by Blakley (1978) to determine the pathway leading to β-oxidation of cyclohexanecarboxylic acid by *Alcaligenes faecalis*. MS was used to identify cyclohex-1-enecarboxylic acid (Figure 1.8, compound b), pimelic acid (Figure 1.8, compound e), and glutaric acid (a β-oxidation intermediate) as their methyl esters. It was found that the intermediates were metabolized as their CoA esters, and that enzymes involved in β-oxidation were induced when the organism was grown on cyclohexanecarboxylic acid. Metabolism of cyclohexanecarboxylic acid by *P. putida* was shown to be similar to that of *A. faecalis* (Blakley and Papish 1982).

That cyclohexanecarboxylic acid can be aromatized was first reported by Kaneda (1974). *Corynebacterium cyclohexanicum* grown on the substrate yielded *p*-hydroxybenzoic acid. It was Taylor and Trudgill (1978) who further elucidated the pathway leading to *p*-hydroxybenzoic acid production, and ring fission, using *Alcaligenes* strain W1. MS identified *p*-hydroxybenzoic acid to be a metabolic product of growth on cyclohexanecarboxylic acid. The pathway (Figure 1.8, path 3) was deduced using enzyme assays, chemical and chromatographic methods. *Alcaligenes* strain W1 was shown to grow on intermediates found along the aromatization pathway, but not on those found along the β-oxidation path. A reaction mixture containing *trans*-4-hydroxycyclohexanecarboxylic acid dehydrogenase and *trans*-4-hydroxycyclohexanecarboxylic acid was extracted, which yielded 4-ketocyclohexanecarboxylic acid (Figure 1.8, compound h), and *p*-hydroxybenzoic acid (Figure 1.8, compound k).

Other pathways to cyclohexanecarboxylic acid aromatization have been proposed by Blakley (1974) and Dutta and Harayama (2001), which are different to that proposed by Taylor and Trudgill (1978). Work on aerobically grown *Arthrobacter* sp., and its cell extracts helped to identify pathway 4 in Figure 1.8 (Blakley 1974). Melting point, paper chromatography, thin layer chromatography, and GC of extracts from *Arthrobacter*

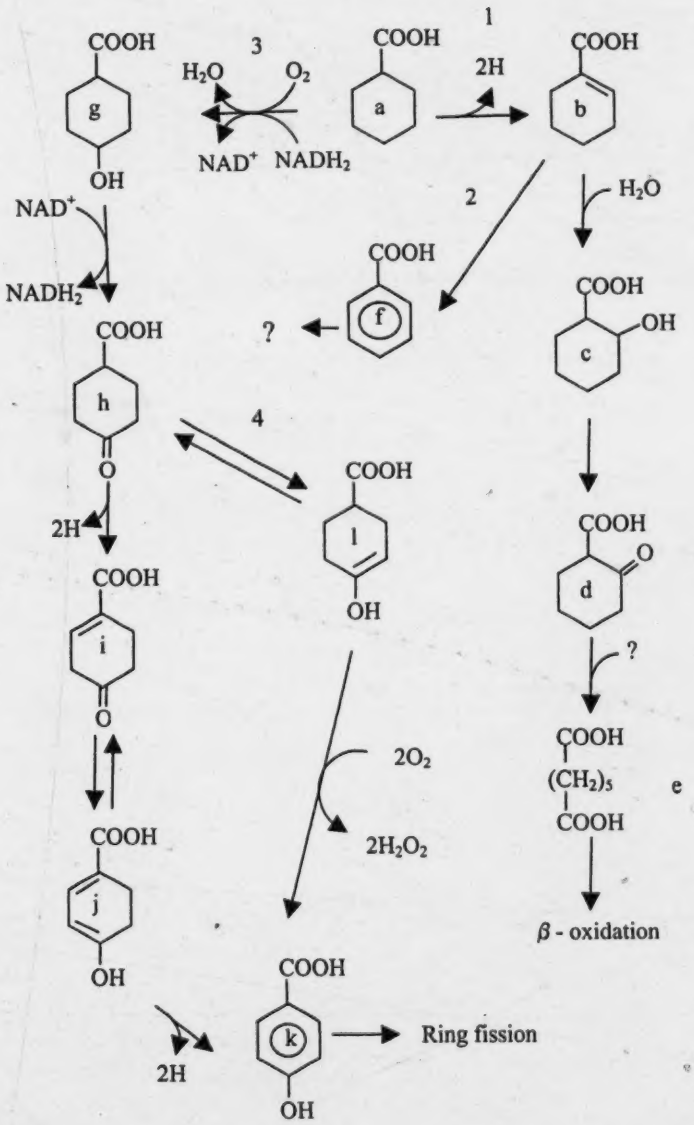


Figure 1.8 Summary of possible cyclohexanecarboxylic acid aerobic biodegradation pathways: (a) cyclohexanecarboxylic acid, (b) 1-cyclohexenecarboxylic acid, (c) *trans*-2-hydroxycyclohexanecarboxylic acid, (d) 2-ketocyclohexanecarboxylic acid, (e) pimelic acid, (f) benzoic acid, (g) *trans*-4-hydroxycyclohexane-carboxylic acid, (h) 4-ketocyclohexanecarboxylic acid, (i) 4-keto-1-cyclohexene-carboxylic acid, (j) 4-hydroxycyclohex-1-enecarboxylic acid, (k) *p*-hydroxy-benzoic acid, (l) 4-hydroxycyclohexane carboxylic acid. Pathways were determined by: (1) Rho and Evans (1975), Smith and Callely (1975), Blakley (1978), Blakley and Papish (1982); (2) Dutta and Harayama (2001), (3) Taylor and Trudgill (1978); (4) Blakley (1974).

grown with methylene blue as terminal electron acceptor indicated the production of *p*-hydroxybenzoic acid (Blakley 1974). Production of hydrogen peroxide (Figure 1.8, path 3) was shown by the oxidation of *o*-dianisidine in the presence of horseradish peroxidase. Dutta and Harayama (2001) used GC-MS to find that cyclohexene-1-carboxylic acid and benzoic acid were intermediates in the catabolism of cyclohexanecarboxylic acid by *Alcanivorax* sp. (Figure 1.8, path 2). They did not attempt to detect further biodegradation of the benzoic acid. GC-MS analysis also shows that in the case of cycloalkanes substituted with alkane chains, the alkane moiety is metabolized by β-oxidation before the ring is processed (Dutta and Harayama 2001).

The biodegradation pathways shown in Figure 1.8 are evidence that biodegradation of cycloalkanoic acids, such as cyclohexanecarboxylic acid is possible. The diversity in proposed pathways, especially the divergence into two main categories, that of ring fission – β-oxidation and that of aromatization – ring fission is proof of the value of such compounds as carbon sources. That is not to say that naphthenic acid components would be as easily biodegradable as the cyclohexanecarboxylic acid surrogate. Unsaturated naphthenic acids, after all, may have more than one ring. Bridged and fused rings may be more of a challenge for microorganisms to degrade. The intermediates shown in Figure 1.8 are also important, in that their presence may indicate biodegradation of larger molecules. On the other hand, such compounds may also inhibit further biodegradation of naphthenic acid components. Moore et al. (2002), for example believe that inhibitory compounds exist in Pond 5 wetland. These inhibitory compounds may be metabolic intermediates from naphthenic acids biodegradation.

1.7.3 Naphthenic acids biodegradation

Although information has been obtained by studying a few surrogate naphthenic acids, it is also important to obtain information on the biodegradability of the naphthenic acids mixtures themselves. Naphthenic acids obtained commercially and from tailings pond extracts have been studied.

According to Holowenko et al. (2001), the alkane moiety in monosubstituted cyclohexanes such as cyclohexanepentanoic acid stimulates methanogenesis, but

commercial and tailings extract naphthenic acids do not. Using tailings naphthenic acids; they found that 150 mg/L in microcosms unamended with hydrogen or acetate had no effect on methanogenesis. However, a 300 mg/L concentration increased the lag time prior to methanogenesis, whereas a 500 mg/L concentration decreased the amount of methane produced by the microcosms. The latter concentrations are higher than those expected in tailings pond waters. Presence of naphthenic acids in the consortium did not increase methanogenesis, indicating that they are not used as carbon sources in methane production (Holowenko et al. 2001).

Lai et al. (1996) used ^{14}C-labeled surrogate naphthenic acids, palmitic and decahydro-2-naphthoic acid, as indicators of Kodak and tailings pond naphthenic acids biodegradation. They showed that biodegradation of the surrogate acids, as indicated by $^{14}CO_2$ production, does not decrease the microcosm toxicity. This in turn was an indication that the commercial and tailings naphthenic acids were probably not biodegraded by the consortium.

Naphthenic acids mixtures extracted from tailings pond, or produced commercially, were biodegraded by aerobic consortia obtained from tailings pond water (Herman et al. 1994). A microbial community acclimated on Kodak salts can degrade this commercial preparation, releasing 48% of the Kodak salts carbon as CO_2. This same community was also able to release 20% of tailings extract carbon as CO_2. Biodegradation of these mixtures corresponded to decreases in toxicity, as determined by Microtox™. There was an observable decrease in Kodak salts naphthenic acids concentration, as determined using GC-FID. However, the same was not observed in tailings extract samples.

Examinations of tailings ponds waters have shown similar trends. MacKinnon and Boerger (1986) observed that toxicity as measured by Microtox™ decreases after 2 years undisturbed storage of tailings. Decreases in toxicity have also been observed by Holowenko et al. (2002) in both Syncrude and Suncor tailings ponds left undisturbed for several years, compared to fresh tailings. The surface water zone of Mildred Lake Settling Basin had a naphthenic acids concentration of 49 mg/L, compared to 36 mg/L in a sample undisturbed for 7 years, and 24 mg/L in a sample undisturbed for 11 years (Holowenko et al. 2002). A similar trend was found in Suncor samples. There,

consolidated tailings release waters contained 68 mg naphthenic acids/L, compared to 44 mg/L in a pond aged for 9 years. These decreases in naphthenic acids concentrations were also related to decreases in toxicity (measured using Microtox™ IC_{50}), and increases in the C22+ cluster. The 11-year-old Syncrude sample had an IC_{50} of >100%, and a C22+ of 22% compared to an IC_{50} of 32% and a C22+ of 12% from Mildred Lake Settling Basin. The Suncor 9-year-old sample had an IC_{50} of >100%, and a C22+ of 20%; compared to an IC_{50} of 64%, and a C22+ of 37% in the consolidated tailings release waters. From this study, decreases in naphthenic acids concentrations were shown to be related to decreases in toxicity and increases in the abundance of compounds with more than 22 carbons (Holowenko et al. 2002).

1.8 Objectives

Extraction of petroleum from sources such as the Athabasca oil sands results in solubilization of naphthenic acids in the hot, caustic water used (Brient et al. 1995). This water is in turn stored in large, artificial storage ponds, such as Syncrude's Mildred Lake Settling Basin, which has a volume of $300x10^6$ m^3 (Leung et al. 2001). Naphthenic acids concentrations in such sites range between 20 to 120 mg/L (Holowenko et al. 2002).

Laboratory studies have shown that naphthenic acids are toxic to fishes such as Caspian round goby, kutum, roach, salmon, and sturgeon (Dokholyan and Magomedov 1984). Using rats as model organisms, mammals also exhibit toxic effects when exposed to naphthenic acids (Rogers et al. 2002b). Toxicity is therefore a concern when planning a reclamation strategy for sites contaminated with these compounds.

Herman et al. (1994) showed that toxicity in microcosms containing naphthenic acids decrease with their mineralization. They showed that 50% of Kodak salts carbon, and 20% of tailings extract carbon is transformed to CO_2 in the presence of tailings pond microorganisms acclimated on naphthenic acids. Such biodegradation is believed to be occurring in tailings ponds waters (Holowenko et al. 2002). Holowenko et al. (2002) found that tailings ponds that were left undisturbed for several years exhibited decreased toxicity (as determined using Microtox™), lower naphthenic acids concentrations, and changes in naphthenic acids composition. An increase in the C22+ cluster, with decrease

in naphthenic acids concentrations was symptomatic of the change in distribution of the acids.

Given the data obtained by Herman et al. (1994) and Holowenko et al. (2002), the present study was performed to test the hypothesis that aerobic microorganisms from tailings pond waters selectively degrade lower molecular weight naphthenic acids. This would explain the increase in proportion of high molecular weight naphthenic acids, with decreasing concentrations, as observed by Holowenko et al. (2002) in the different tailings ponds. Herman et al. (1994) demonstrated that toxicity was related to naphthenic acids concentrations. Another implication of their data is that not all naphthenic acid components are equally toxic, since Holowenko et al. (2002) demonstrated the recalcitrance of compounds with C22+, and decreases in toxicity despite this. The main objectives of the project were therefore:

1) To characterize naphthenic acids extracted from different oil sands ores.
This would give an indication as to the distribution of naphthenic acid components upon bitumen extraction. Although biodegradation of naphthenic acids from tailings pond wastewaters is not reported here, studying naphthenic acids distribution from freshly extracted samples allows subsequent comparisons to biodegraded samples. This can help determine if certain fractions are more easily biodegraded.

2) To study the biodegradation of two commercial naphthenic acids preparations: Kodak salts, and Merichem acids.
The three-dimensional fingerprint of the initial mixture will be compared to mineralized samples to determine any changes. These changes will be compared to mineralization, as well as decreases in toxicity, and naphthenic acids concentration.

To help meet these objectives, several goals were set:

1) To develop statistical methods, which would allow comparison of three-dimensional fingerprints of derivatized naphthenic acids.

2) To follow the biodegradation of two commercial naphthenic acids mixtures (Kodak, and Merichem) by aerobic microcosms acclimated from tailings water. This will involve measuring naphthenic acids concentrations, mineralization (CO_2 release), toxicity, and changes in component distribution.

3) To determine changes in naphthenic acids component distribution, the GC-MS method developed by St. John et al. (1998) and applied by Holowenko et al. (2002) will be used. An increase in the proportion of compounds in the C22+ cluster would indicate that lower molecular weight components are preferentially degraded.

4) To follow the changes in concentrations, an analytical method using NPH and HPLC as developed by Miwa et al. (1985) will be modified to measure naphthenic acids concentrations.

5) To measure the decrease in toxicity resulting from naphthenic acids biodegradation using Microtox™.

1.9 Thesis Overview

This thesis is organized such that Chapters 3 and 4 explain the statistical methods used to compare naphthenic acid three-dimensional graphs. Chapter 3 is the initial attempt, using an independent samples t-test, while Chapter 4 uses pairwise multiple comparisons, and is concerned with comparing 15 oil sands ore samples. Chapters 2 and 5 elaborate on methods used in the biodegradation experiment (Chapter 6). Chapter 2 explains the HPLC method used to determine changes in naphthenic acids concentrations in laboratory cultures. Chapter 5 explains the liquid-liquid extraction procedure chosen to prepare the naphthenic acids for GC-MS analysis. This Chapter utilizes the statistical tools from Chapter 3 to determine differences in the fraction of naphthenic acids extracted when different solvents were used. Chapter 6 illustrates the biodegradation of Kodak salts and Merichem naphthenic acids by mixed microbial cultures from Mildred Lake Settling Basin, which have been acclimated on these same acids. This Chapter is a

synthesis of the information from Chapters 2 through 5. Here, naphthenic acids concentrations were determined using HPLC, the naphthenic acids distribution of both preparations were determined and compared in Chapters 3 and 4, and the extraction efficiency and limitations were determined in Chapter 5. Chapter 7 is a summary of the information gleaned from using the method developed by St. John et al. (1998).

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2. DEVELOPMENT OF AN HPLC METHOD TO ESTIMATE NAPHTHENIC ACIDS CONCENTRATIONS IN LABORATORY CULTURES[♣]

2.1 Introduction

Naphthenic acids are complex mixtures of alkyl-substituted acyclic and cycloaliphatic carboxylic acids with the general chemical formula $C_nH_{2n+Z}O_2$, where n indicates the carbon number and Z is zero or a negative, even integer that specifies a homologous series. Values of n range from about 5 to 33, giving molecular weights between 100 and 500 g/mol. Typical naphthenic acids structures are given in Chapter 1, Figure 1.1. These acids are found in many petroleums (Lochte and Littman 1955, Fan 1991, Brient et al. 1995, Slavcheva et al. 1999, Jones et al. 2001) and cause corrosion during oil refining (Slavcheva et al. 1999). Naphthenic acids may be present in wastewaters at petroleum refineries (Dzidic et al. 1988, Wong et al. 1996) and they are abundant in the process-affected waters at the oil sands extraction plants in Alberta, Canada (Holowenko et al. 2002, Rogers et al. 2002a, Schramm et al. 2000). Currently, most of the extraction of bitumen from oil sands is based on the Clark caustic hot water extraction process, in which the oil sand ore is digested with warm water and sodium hydroxide (Schramm et al. 2000). This alkaline extraction dissolves the naphthenic acids and leaves them in the aqueous phase as naphthenates. The oil sands companies do not release any extraction wastes from their property leases, so that the process-affected waters and fluid tailings are contained on site, primarily in large settling ponds. The resulting process-affected waters have been shown to have naphthenic acids concentrations in the range of 40 to 120 mg/L (Holowenko et al. 2000, 2001; Schramm et al. 2000).

Several studies have shown that naphthenic acids are toxic to different organisms (Dokholyan and Magomedov 1983, MacKinnon and Boerger 1986, Dorn 1992, Rogers et

[♣]A version of this chapter has been published. Clemente, J.S., T.-W. Yen, and P.M. Fedorak. 2003. J. Environ. Eng. Sci. 2: 177-186.

al. 2002b). Natural aging of oil sands tailings water reduces its toxicity (Holowenko et al. 2002, MacKinnon and Boerger 1986) and microbial activity in laboratory cultures reduces the toxicity of naphthenic acids (Herman et al. 1994).

The analyses of naphthenic acids present a major challenge. Many workers have applied various mass spectrometry methods (Dzidic et al. 1988, Fan 1991, Wong et al. 1996, Hsu et al. 2000) and gas-chromatography-mass spectrometry (GC-MS) methods (St. John et al. 1998, Holowenko et al. 2001, 2002) to attempt to qualitatively characterize the types of compounds in naphthenic acids from different sources. Quantitatively, naphthenic acids in crude oils and distillates are commonly measured by titrating with KOH to determine the neutralization number, using ASTM methods D664 or D974 (Brient et al. 1995, Slavcheva et al. 1999). Jones et al. (2001) recently described a GC-MS method for estimating the naphthenic acids content of crude oils. There appears to be only one method that has been used for quantitatively measuring naphthenic acids in aqueous samples. This was developed by Jivraj et al. (1995) at Syncrude Canada Ltd., one of the oil sands companies. This method has been described in detail by Holowenko et al. (2001) and Rogers et al. (2002a). Briefly, the method involves acidifying the aqueous sample, extracting the naphthenic acids into dichloromethane, concentrating the extract, and analyzing the concentrate by Fourier-transform infrared (FT-IR) spectroscopy. The absorbances at 1743 and 1706 cm^{-1}, characteristic of carboxylic acids, are measured in the IR spectrum.

To aid in the studies of naphthenic acids biodegradation in laboratory cultures, I sought a simple, fast and reliable method to measure naphthenic acids concentrations in an aqueous medium. The titration method and the FT-IR method essentially quantify the carboxylic groups in the naphthenic acids. Thus, I took the same general approach by derivatizing the carboxyl groups with 2-nitrophenylhydrazine (NPH), a reagent that has been used extensively by Miwa and coworkers (Miwa 1985, 2000, Miwa and Yamamoto 1987, 1988, Miwa et al. 1985, 1987, 1990). I then separated the derivatized naphthenic acids from the excess reagents by high performance liquid chromatography (HPLC). Because of the complexity of mixtures of naphthenic acids, the derivatized compounds eluted from the column as a "hump" of unresolved compounds. Comparing the areas

under the "humps" in standard preparations and in culture supernatants allowed measurements of the depletion of naphthenic acids from aerobic microbial cultures.

2.2 Materials and Methods

2.2.1 Naphthenic acids and carboxylic acid standards

Three commercially available preparations of naphthenic acids were used in this study. Kodak naphthenic acids sodium salt (lot B14C) and Kodak naphthenic acids (lot 115755A) were purchased from The Eastman Kodak Company (Rochester, NY). Refined naphthenic acids were a gift from Merichem Chemicals and Refinery Services LLC (Houston, TX).

Several accurately weighed samples of the naphthenic acids sodium salts were dissolved in MilliQ water, and the sodium concentration was determined by atomic absorption spectroscopy in the Limnology Laboratory, Department of Biological Sciences. These results showed that the commercially available salt preparation contained 9% (w/w) sodium. A sample of Kodak acids was analyzed for carbon content in the Microanalytical Laboratory, Department of Chemistry.

Naturally occurring naphthenic acids were extracted from 160 L of water collected from the Mildred Lake Settling Basin at the Syncrude oil sands extraction site. Details of the extraction method are given by Holowenko et al. (2001). The extraction provided 1.2 L of Mildred Lake Settling Basin naphthenic acids (MLSBF) in an alkaline solution with a concentration of 3100 mg/L as determined by FT-IR spectroscopy (Holowenko et al. 2001).

The following carboxylic acids that fit the empirical formula of naphthenic acids ($C_nH_{2n+Z}O_2$) were purchased: 1-methylcyclohexanecarboxylic acid, cyclohexane-propanoic acid, *trans*-4-pentylcyclohexanecarboxylic acid, lauric acid, palmitic acid, cyclohexanebutanoic acid, (all from Aldrich Chem. Co. Milwaukee, WI), stearic acid (BDH Chemicals Ltd., Poole, England), and 5β-cholanic acid (Sigma Chemicals Co., St. Louis, MO). Each of these was individually derivatized and analyzed by HPLC to

determine their elution times. One biodegradation study used *trans*-4-pentyl-cyclohexane carboxylic acid as a model naphthenic acid.

To facilitate dissolution, concentrated solutions of the naphthenic acids, sodium salts or *trans*-4-pentylcyclohexane carboxylic acid were prepared in 0.25 M or 0.1 M NaOH. Various concentrations of solutions in 0.25 M NaOH were diluted in modified Bushnell-Haas mineral salts medium (Wyndham and Costerton 1981) to prepare standards for calibration curves. Appropriate volumes of the concentrated solutions of naphthenic acids in 0.1 M NaOH were added to the culture medium for biodegradation studies.

2.2.2 Culture methods

Naphthenic acids-degrading enrichment cultures were established on each of the following three naphthenic acids preparations: the Kodak naphthenic acids sodium salts, the refined Merichem naphthenic acids, and the MLSBF. Water from Mildred Lake Settling Basin was used as the source of microorganisms for each of the three naphthenic acids preparations. The enrichment cultures were established in 500-mL Erlenmeyer flasks that contained 200 mL of sterile modified Bushnell-Haas mineral salts medium inoculated with 20 mL of the Mildred Lake Settling Basin water. The naphthenic acids preparation served as the sole carbon source, and each preparation was added at 100 mg/L. The final pH was approximately 7.0. Cultures were incubated in the dark on a shaker at room temperature (about 21 °C) and transferred to fresh medium and the corresponding naphthenic acids preparation every 1 to 2 weeks. Cultures had been acclimated for 11 months prior to the experiment.

Cultures were established for the evaluation of the HPLC method to monitor the biodegradation of *trans*-4-pentylcyclohexanecarboxylic acid, the Merichem acids, and the MLSBF, each at an initial concentration of 100 mg/L (Section 2.3.4). Stock solutions (2000 mg/L) of each of the commercially available substrate were prepared in 0.1 M NaOH. Viable cultures and sterile controls were established for each of the three substrates in 500-mL Erlenmeyer flasks. Each viable culture consisted of 170 mL of sterile modified Bushnell-Haas mineral salts medium, 10 mL of filter-sterilized stock

substrate solution (or 7.1 mL of the MLSBF naphthenic acids preparation), and 20 mL of the corresponding enrichment culture. The medium that contained *trans*-4-pentyl-cyclohexanecarboxylic acid was inoculated with 10 mL of the culture enriched on Kodak salts and 10 mL of the culture enriched on the Merichem acids. The controls contained 190 mL of nitrogen-free sterile modified Bushnell-Haas mineral salts medium (in this case, no ammonium or nitrate was added) and 10 mL of the filter-sterilized stock substrate solution or 7.1 mL of the MLSBF naphthenic acids preparation. One sterile control and one viable culture were prepared for each naphthenic acids preparation.

Control and culture flasks were incubated in the dark at room temperature with shaking to provide aeration. Every few days, 3-mL samples were removed from each control and viable culture. These were stored at 4 °C in 1-dram glass vials until they were analyzed by HPLC. In preparation for HPLC analysis, the stored samples were mixed thoroughly by vortexing, then 1-mL portions were removed and centrifuged for 10 min at 14 000 rpm in an Eppendorf model 5415c microfuge (Brinkman Instruments Inc., Westbury, NY) to pellet cells. A portion of the supernatant was then derivatized as outlined below.

An experiment was done to determine if the loss of naphthenic acids from a viable culture was accompanied by mineralization (the release of CO_2). This experiment was done using Kodak acids in 120-mL Erlenmeyer flasks that were modified by the addition of a side-arm that was sealed with serum stopper. The total volume of the flask was 140 mL. During incubation, the top of the flask was also sealed with a serum stopper to make a gas-tight incubation system so that none of the microbially produced CO_2 escaped. Headspace gas and the liquid culture medium were sampled using a syringe, without removing the serum stoppers.

A stock solution of the Kodak acids was prepared at 2000 mg/L in 0.1 M NaOH. Each mineralization culture was prepared by adding 2 mL of the stock solution to 36 mL of modified Bushnell-Haas medium and 2 mL of viable inoculum from the culture enriched on the Kodak salts. This gave a final volume of 40 mL with the naphthenic acids at 100 mg/L. Fifteen replicate cultures were prepared for each experiment. The sterile controls contained 36 mL of medium, 2 mL of the stock naphthenic acids solution, and 2 mL of the heat-killed enrichment culture.

The viable cultures and controls were incubated in the dark on a shaker at 200 rpm at room temperature. At each sampling time, a viable culture and one control flask were destructively sampled for naphthenic acids analysis by HPLC, and for bicarbonate and CO_2 analysis by GC to demonstrate mineralization. Samples collected for naphthenic acids analyses were stored at –20 °C until HPLC analyses were done. The inorganic forms of carbon were measured using the method of Bressler et al. (1999). Briefly, 2 mL of liquid and 5 mL of airspace (5% of the volume of each phase) were sampled from the sealed culture flask using a syringe. These samples were injected into a sealed 40-mL serum bottle containing 2 mL of 2 M sulfuric acid. After equilibrating overnight, a 0.5-mL sample of the headspace gas was analyzed by GC (Bressler et al. 1999). Standards for calibration curves were prepared by adding known amounts of $NaHCO_3$ in Tris buffer (pH 8) into empty culture flasks. These were sealed, then sampled and treated in the same manner as the viable cultures.

2.2.3 Derivatization method

Because alkaline solutions of the naphthenic acids were added to the culture medium, I used the method of Miwa (2000) which was devised for derivatizing carboxylic acids in alkaline foods and beverages. Briefly, 100 µL of naphthenic acid sample (or standard solution) were mixed with 200 µL of acidic, 0.02 M NPH (ICN Biomedicals Inc. Aurora, OH) solution and 200 µL of 1-ethyl-3-(3-dimethylamino-propyl)carbodiimide hydrochloride (EDC, Sigma Chemicals Co., St. Louis, MO) solution, in a sealed 1.5-mL, screw-cap vial. The acidic NPH solution was prepared by dissolving 60 mg of NPH in 10 mL of ethanol and mixing this with 10 mL of 0.1 M HCl. The EDC solution was prepared by dissolving 480 mg of EDC in 10 mL of 95% ethanol and mixing this with 10 mL of 3% pyridine in 95% ethanol.

The reaction mixture (containing sample, acidic NPH, and EDC) was incubated in a hot water bath at 60 °C for 20 min. Vials were removed from heat and 100 µL of 69 mM KOH (made in 80% (v/v) methanol in water) were added. The reaction mixture was then incubated in the water bath for another 15 min at 60 °C. The derivatized samples were then cooled by placing them on cold water prior to HPLC analysis.

2.2.4 HPLC method

All HPLC analyses were performed using an Agilent (Wilmington, DE) 1100 Series HPLC with an autosampler, thermostated column compartment, and a UV-Visible diode array detector, connected to an Agilent Chemstation operating with software for LC 3D systems. The HPLC was fitted with a low-volume column inlet filter (2 μm pore size) to protect the analytical LiChrospher 100 RP-18 column (5 μm particle size, 125 mm x 4 mm, Agilent). The column temperature was maintained at 40 °C. The solvent flow rate was 1.5 mL/min. Two solvents were prepared. Solvent A was HPLC grade methanol (Fisher Chemicals, Fairlawn, NJ) that contained 90 μL of 0.185 M phosphoric acid aqueous solution per liter of methanol. Solvent B was 28 μM phosphoric acid made in MilliQ water. For the first 2 min after a 60-μL sample injection, the mobile phase consisted of 70% solvent A and 30% solvent B. Thereafter, the mobile phase was 100% solvent A.

The detector was set at 400 nm (with a bandwidth of 10 nm), with the reference wavelength of 510 nm (with bandwidth of 60 nm). The unreacted derivatizing agents eluted within 2.9 min, and all of the absorbance between 2.9 min and 6.0 min was summed yielding the area of the "hump" due to derivatized naphthenic acids. The total run time was 7 min.

2.3 Results

2.3.1 Identifying the peaks eluting from the derivatized naphthenic acids mixture

Figure 2.1 shows the output of the HPLC detector monitoring the column effluent at 400 nm. When no sample was injected into the HPLC, there was a small disturbance in the baseline caused by the change from the mixture of solvents to 100% solvent B (Figure 2.1D). The mobile phase composition was designed so that when a reagent blank (with no naphthenic acids) was injected, most of the unreacted reagents elute quickly (in <3 min) as shown in Figure 2.1C. The absorbance of the material in the reagent blank that elutes between 3 and 4 min was always higher than observed with no sample injected (i.e.

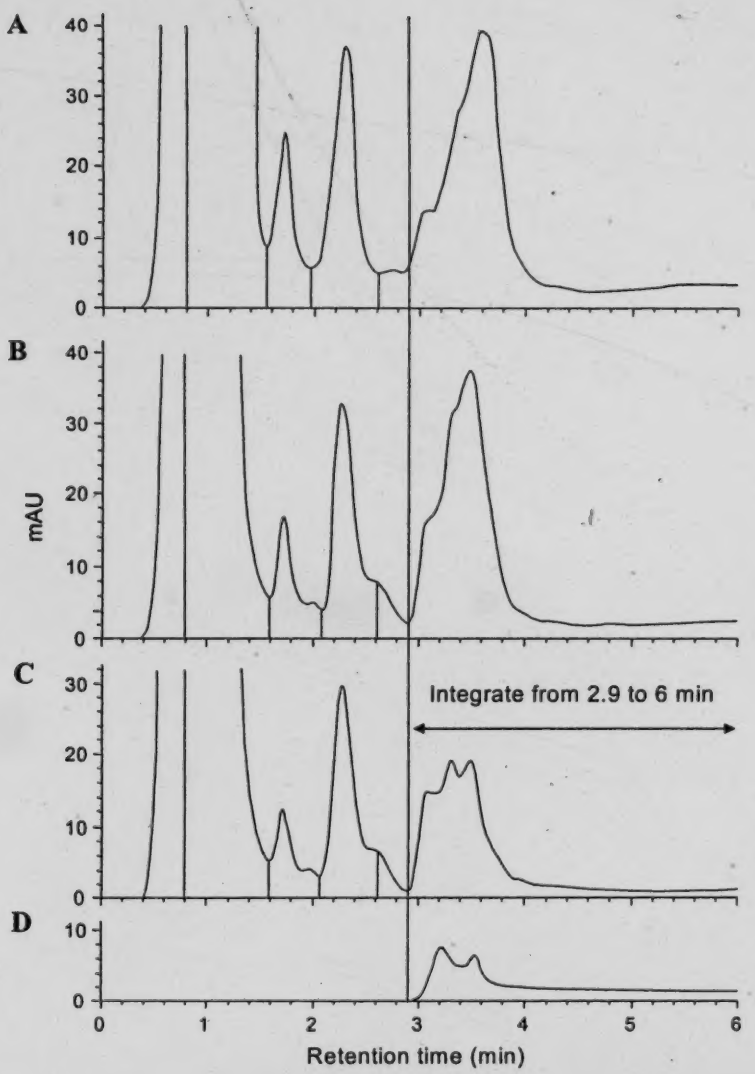

Figure 2.1 HPLC detector output monitoring absorbance at 400 nm: (A) injection of 60 µL of derivatized MLSBF naphthenic acids (100 mg per liter in modified Bushnell-Haas mineral salts medium), (B) injection of 60 µL of derivatized standard containing 100 mg Kodak naphthenic acids per liter of modified Bushnell-Haas mineral salts medium, (C) injection of 60 µL of reagent blank with no naphthenic acids in modified Bushnell-Haas mineral salts medium, (D) injection of 0 µL of sample.

Figure 2.1D). A larger peak between 3 and 4 min was observed when derivatized naphthenic acids (or model naphthenic acids) were injected (Figure 2.1B). Analysis of the

MLSBF naphthenic acids yielded essentially the same chromatogram (Figure 2.1A) as that obtained with a commercially available naphthenic acid preparation (Figure 2.1B).

Comparing Figures 2.1C and 2.1D shows that most of the unreacted derivatizing reagents elute before 2.9 min. Thus, integration of the hump of derivatized naphthenic acids was started at 2.9 min, where a valley was typically observed in the chromatogram (Figure 2.1). To estimate the time at which integration should be stopped, eight pure compounds, fitting the general formula for naphthenic acids ($C_nH_{2n+Z}O_2$), were individually derivatized and analyzed by HPLC to determine their elution times (Section 2.3.2). The derivatives of 1-methyl-1-cyclohexanecarboxylic acid (underivatized molecular weight 142.2) and cyclohexanepropanoic acid (156.2) eluted with the excess derivatizing agent (prior to 2.9 min). Thus, these compounds were not considered in the evaluation of the elution times and molecular weights.

2.3.2 Using carboxylic acids to determine elution times

Figure 2.2 shows the elution times for cyclohexanebutyric (underivatized molecular weight 170.2), *trans*-4-pentylcyclohexanecarboxylic (198.3), lauric (200.3), palmitic (256.4), stearic (284.5), and 5β-cholanic (360.6) acids. There was a strong linear relationship between molecular weights of the underivatized acids and their elution times (R = 0.998). Extrapolation using the regression equation indicated that compounds with molecular weights of 500 g/mol would elute at about 6 min. Very few naphthenic acids detected by mass spectrometry or GC-MS have molecular weights above 500 g/mol (Fan 1991, Dzidic et al. 1988, Holowenko et al. 2002). Therefore, the integration of the HPLC analysis was terminated at 6 min as shown in Figure 2.2.

2.3.3 Analysis of commercial and tailings extract naphthenic acids

Calibration curves prepared with single injections of different concentrations of two commercially available naphthenic acids and with the MLSBF naphthenic acids are shown in Figure 2.3. High linear correlation coefficients (R typically >0.99) were obtained for each naphthenic acids preparation, and the three different sources of acids

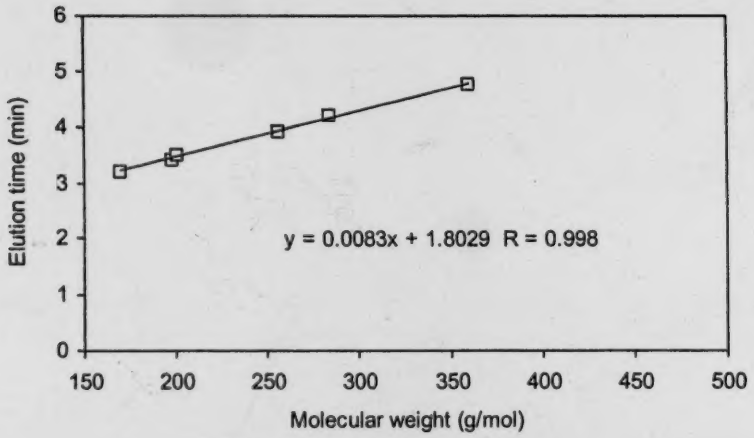

Figure 2.2 The HPLC elution times of six model naphthenic acids plotted against their molecular weights.

gave fairly similar slopes and intercepts. Commonly, the y-intercepts for these calibration curves had values between 500 and 900 area counts. Figure 2.1C shows that the reagent blank yields a "hump" which is integrated, thus the regression equations would be expected to cross the y-axis at some positive value.

The precision of the HPLC method was very high. Standards of the Kodak acids were prepared at concentrations of 10, 50 and 100 mg/L, and derivatized. Five injections of each reaction mixture were analyzed by HPLC, and the relative standard deviations of the area counts were only 2.1%, 0.7%, and 1.7%, respectively. Samples were removed from the shake-flask cultures of the biodegradation experiment at various times, and stored for several days (0 to 12 days) at 4 °C prior to analyses by HPLC. After four to six sampling times, all of the stored samples were analyzed. Subsequently, additional samples were collected as the cultures were incubated longer, and these too were analyzed at later dates. Each time the samples were analyzed, new calibration curves with the appropriate naphthenic acids were prepared. Table 2.1 summarizes the results from some of the analyses of sterile controls that contained the Merichem acids or the MLSBF naphthenic acids done on different dates. The agreement among some of the replicate analyses was very good. For example the analyses of the day 1 sample taken from the sterile control that contained Merichem naphthenic acids gave concentrations of 92, 89, and 87 mg/L (Table 2.1). However, there were some replicate analyses that gave poor

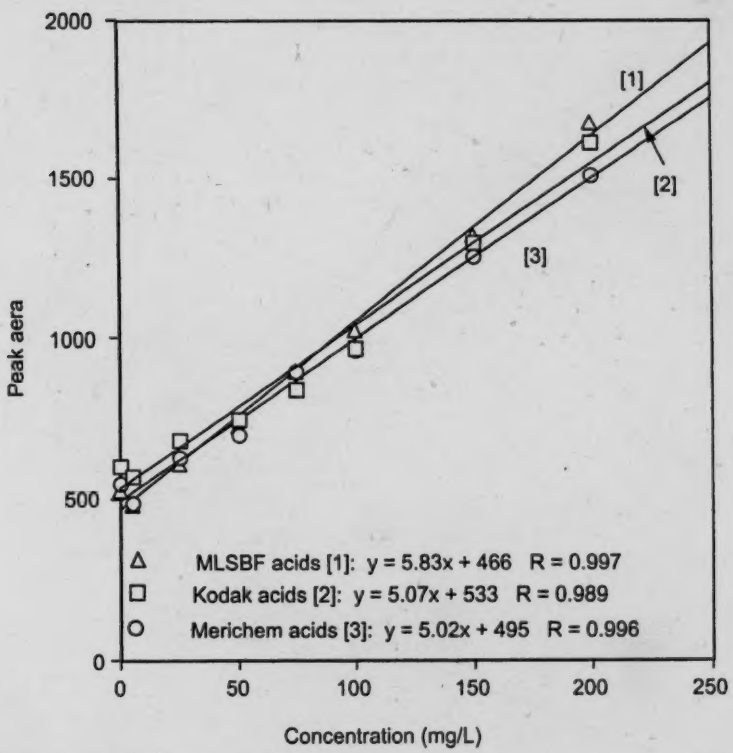

Figure 2.3 Calibration curves with two commercially available naphthenic acids and the MLSBF naphthenic acids.

day-to-day reproducibility. For example, the samples that were taken on days 1, 2, and 3 from the sterile control that contained MLSBF naphthenic acids showed poor reproducibility. The differences between the replicate analyses varied by up to 26 mg/L. Considering all of the data in Table 2.1, the relative standard deviations of the replicate analyses varied from 2.8% to 12.6% (Table 2.1). The mean relative standard deviation was 6.7%.

The samples from the viable cultures shown in Figure 2.4 were stored in a refrigerator (at 4 °C) and analyzed on three different occasions, in the same manner as was done with the controls shown in Table 2.1. As with the controls, the measured naphthenic acids concentrations in the viable cultures varied from day-to-day (data not shown). However, the concentrations for a given sample did not continually decrease with increased storage time. For example, the three sequential analyses of a sample

Table 2.1 Results from the analyses of sterile controls that contained the Merichem acids or the MLSBF naphthenic acids done on different dates.

		Concentration (mg/L)					
Naphthenic acids	Incubation time (day)	First analysis	Second analysis	Third analysis	Mean	Std. Dev	Relative Std Dev (%)
Merichem	0	108	93	96	99	7.9	8.0
	1	92	89	87	89	2.5	2.8
	2	86	96	103	95	8.5	9.0
	3	86	88	91	88	2.5	2.8
	4	98	96	106	100	5.3	5.3
	9	86	84	92	87	4.2	4.8
MLSBF	0	108	115	112	112	3.5	3.1
	1	116	90	105	104	13.1	12.6
	2	118	96	108	108	11.0	10.3
	3	115	92	110	106	12.1	11.4
	4	107	104	112	108	4.0	3.8
	9	109	100	na*	104	6.4	6.1

* not analyzed

removed from the Merichem acids-containing culture after 4 days of incubation gave naphthenic acids concentrations of 58, 53 and 63 mg/L, respectively. Thus, there was no evidence that biodegradation of the naphthenic acids continued in the stored viable culture samples during refrigeration. In total, 14 stored samples from the viable cultures were analyzed on three different occasions. The relative standard deviations of the replicate analyses varied from 1.1% to 18.6%, and the mean relative standard deviation was 11.2%.

I have not been able to trace the cause for these day-to-day variations in the measured concentrations. However, the "humps" in the chromatograms (Figure 2.1C and 2.2D) that appear when no naphthenic acids are injected likely contribute to these variations. To consider the possibility that materials from previous analyses were being washed off the column with subsequent injections, a solution of 70% solvent A and 30% solvent B was prepared, and 60-μL portions of this were injected into the HPLC. Fourteen consecutive injections were made, and the area counts ranged from 265 to 449

with a mean of 343 and a relative standard deviation of 20%. If residual materials were being washed off the column, each successive injection should have given a lower area count. However, no trend was observed in the areas of the "humps", so there was no indication that residual materials in the HPLC were the cause of these "humps" when only HPLC solvents were injected.

The area counts of the reagent blanks were generally quite reproducible on a given day, but these varied from day-to-day, and with minor changes to the HPLC system. For example, the area counts for the reagent blanks (0 mg/L), analyzed on different days, ranged from 529 to 603 (Figure 2.3). On another occasion, five injections of a reagent blank were done, and the mean area count was 820 and the relative standard deviation was 2.3%. This precision was comparable to that observed with Kodak acids at concentrations of 10, 50 and 100 mg/L (discussed above). After a six-port valve was installed in our HPLC system (to allow rapid switching between HPLC columns for different analyses), the area counts for the reagent blank rose to about 900. Despite the changes in the area count of the reagent blank, which caused the y-intercept to change, the calibration curves were linear. The high area counts of the reagent blanks, limits the ability to measure low concentrations of naphthenic acids. My experience suggests that naphthenic acids concentrations of <15 mg/L cannot be accurately determined by this HPLC method.

The differences (greater than the desired maximum of 5% relative standard deviation) observed in the results obtained from analysis of the same sample on different days made it difficult to state the absolute concentration of naphthenic acids in the cultures at various times during the biodegradation study. Thus, the data from the biodegradation experiments were presented as the ratio C_t/C_0, the concentration at any time (C_t) divided by the original concentration (C_0) determined by HPLC analyses. All of the stored samples from the control and viable culture with a given naphthenic preparation were analyzed on one day at the end of the experiment to yield the C_t/C_0 values for that experiment.

2.3.4 *Naphthenic acids biodegradation monitored using HPLC*

Figure 2.4 summarizes the results from the biodegradation experiment. Each panel shows the C_t/C_0 values for the sterile control (open symbols) and the viable culture (solid symbols). Each C_t value was the result of a single HPLC analysis of a sample taken from the control or viable cultures. After about an 8 day lag period, the mixed enrichment culture degraded the pure compound, *trans*-4-pentylcyclohexanecarboxylic acid (Figure 2.4A). This was the most extensive decrease in the substrate concentration observed among the viable cultures. The relative concentrations of naphthenic acids also decreased in each of the other two cultures, with a decrease of about 30% observed with the Merichem acids (Figure 2.4B) and of about 40% observed with the MLSBF naphthenic acids (Figure 2.4C).

The last five C_t/C_0 values for each control and viable culture (Figure 2.4) were arbitrarily chosen to determine whether there was a statistical difference between the final amounts of naphthenic acids. The mean C_t/C_0 values from the viable cultures were compared to the mean C_t/C_0 values of the corresponding sterile controls. In all three cases (Figure 2.4) the average C_t/C_0 values from the viable cultures were significantly less ($P<0.001$) than the average from the C_t/C_0 values from the sterile controls, based on a two-sided, unpaired t-test assuming equivalent variance. For example, the average C_t/C_0 values of the last five sampling times for the Merichem acids (Figure 2.4B) were 0.95 for the sterile control and 0.71 for the viable culture (giving $P=0.0006$). These results demonstrate a decrease in the amount of naphthenic acids in the viable cultures, indicating that biodegradation can be followed by the HPLC method devised in this study.

The data in Figure 2.5 illustrate that the removal of naphthenic acids from culture medium is accompanied by mineralization. The cultures used in this mineralization experiment were incubated in sealed flasks with 100 mL of air space, 21% of which is initially oxygen, and with 40 mL of medium that contained 100 mg/L naphthenic acids. The Kodak naphthenic acids were 73.9% carbon by weight. Thus, each culture contained 0.25 mmol carbon. Using a GC-MS method to characterize the Kodak acids, Chapter 3 will show that 70% of the acids in this mixture contain 13 carbon atoms or less. Thus,

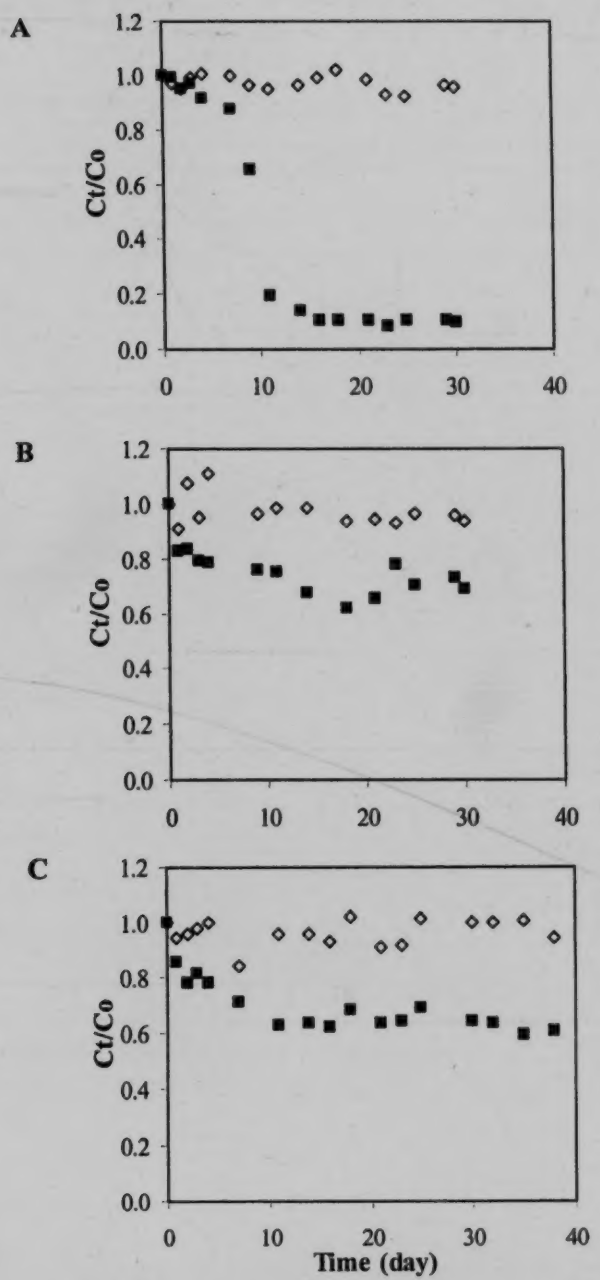

Figure 2.4 Summary of results from the biodegradation experiments with (A) *trans*-4-pentylcyclohexanecarboxylic acid, (B) Merichem acids, and (C) MLSBF naphthenic acids. Open symbols are results from the sterile controls, solid symbols are results from the viable cultures.

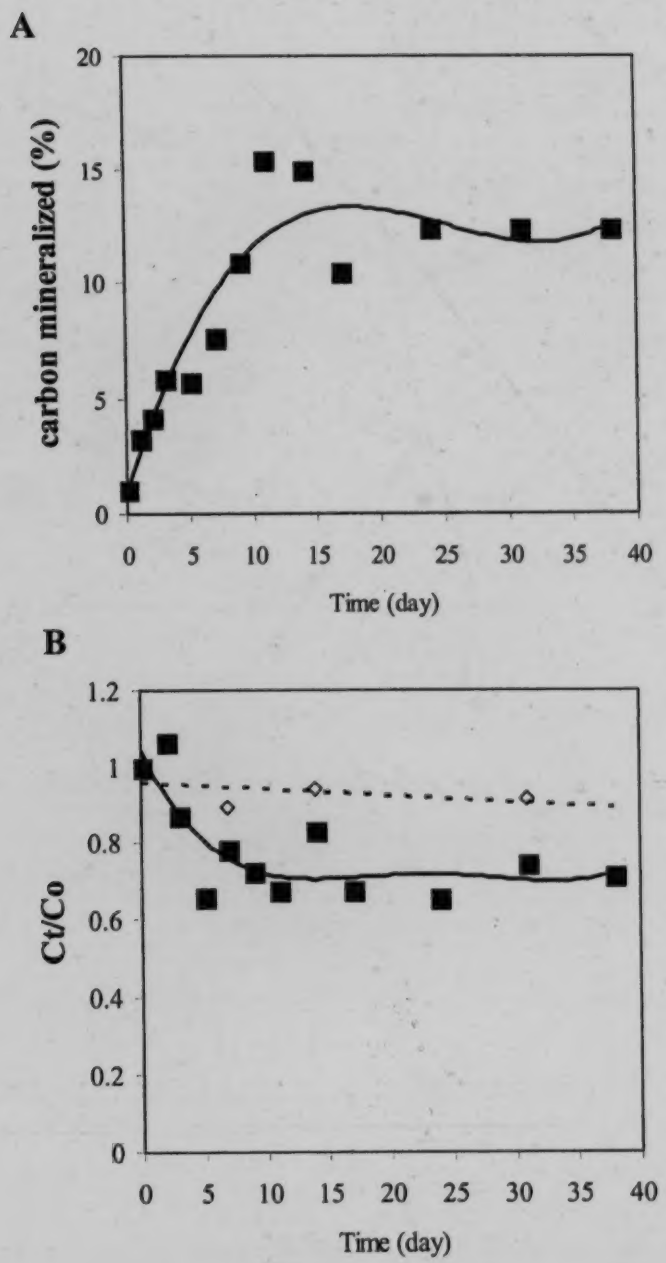

Figure 2.5 Summary of the mineralization experiment with Kodak acids showing (A) the proportion of the naphthenic acids carbon found as CO_2 and bicarbonate, and (B) the changes in naphthenic acids concentrations. Open symbols contain heat-killed cultures, closed symbols contain viable cultures (n=1).

the amount of O_2 required for complete mineralization of these naphthenic acids can be estimated from the equation $C_{13}H_{26}O_2 + 18.5O_2 \rightarrow 13CO_2 + 13H_2O$. From this, for each mmol of carbon, 1.42 mmol of O_2 is required for complete mineralization. Thus, in each culture flask, 0.34 mmol O_2 is needed for complete mineralization. Assuming standard temperature and pressure, this is equivalent to 7.6 mL of O_2. However, each sealed flask contained about 21 mL of O_2, so oxygen was not limiting in the sealed flasks.

Over the 38 day incubation period, the naphthenic acids concentration remained essentially constant in the sterile controls, while there was about a 30% decrease in the C_t/C_o concentration of Kodak acids in the viable cultures (Figure 2.5B). At each sampling time shown in Figure 2.5, one replicate flask was destructively sampled. Thus, some of the variability in the observed results would be due to differences among the replicate cultures. The greatest decrease in naphthenic acids concentration occurred during the first 10 days of incubation. This decrease was accompanied by the formation of inorganic carbon species (bicarbonate and CO_2) in the viable cultures, shown as mineralization in Figure 2.5A. These results were corrected for background CO_2 and bicarbonate measured in the sterile control, which remained unchanged over the 38 days. The 30% decrease in naphthenic acids concentration occurred with about 13% of the carbon in the Kodak acids being mineralized. This is consistent with the general assumption that aerobic heterotrophic activity releases about 50% of the substrate carbon as CO_2 (Maier et al. 2000).

2.4 Discussion

From the onset of this investigation, no attempt was made to separate and quantify individual compounds in the complex naphthenic acids mixtures. This approach used the well-documented method with NPH to derivatize fatty acids (Miwa 1985, 2000, Miwa and Yamamoto 1987, 1988, Miwa et al. 1985, 1987, 1990). I then devised an HPLC method that would elute the derivatized compounds as a "hump" that could be integrated (Figure 2.1). Recently, Jones et al. (2001) took a similar approach to quantify naphthenic acids by derivatizing the acids to their methyl esters, and then analyzing these by GC using a flame ionization detector. This yielded an unresolved "hump" in the

chromatogram, which was integrated between 5 to 80 min. Similarly, Herman et al. (1994) used the changes in the areas under "humps" in gas chromatograms to detect changes in the naphthenic acids in laboratory biodegradation studies.

The analyses of eight pure carboxylic acids that are model naphthenic acids indicated that the HPLC method will not detect some low molecular weight naphthenic acids that have a retention time <2.9 min. For example, the derivatized cyclohexane-propanoic acid eluted with the unreacted reagents, and could not be quantified. This indicates that acids with molecular weights approximately ≤156 g/mol would not be included in the hump that is integrated after 2.9 min. This corresponds to naphthenic acids of about $n \leq 9$ (Holowenko et al. 2002). Results from GC-MS analyses of naphthenic acids indicate that the relative abundance of ions corresponding to $n \leq 9$ is small. GC-MS analyses of naphthenic acids from two oil-sands tailings ponds (designated MLSBF and Pit 5) showed that about 6% of the ions detected corresponded to acids with $n \leq 9$ (Chapter 3). Similarly, 8% (unpublished results) and 6% (Holowenko et al. 2002) of the ions corresponding to acids with $n \leq 9$ were detected in the Merichem acids and naphthenic acids extracted from an oil sands ore, respectively. Thus, only a small portion of the acids in naphthenic acids mixtures would not be quantified as part of the "hump" produced by this HPLC method.

Several investigations have focused on the laboratory biodegradation of naphthenic acids and model compounds that resemble naphthenic acids (Herman et al. 1994, Lai et al. 1996, Holowenko et al. 2001). Herman et al. (1994) showed that *trans*-4-pentylcyclohexanecarboxylic acid was mineralized to various extents by mixed microbial cultures that originated from the oil sands tailings waters. Up to 26% of the carbon from this model naphthenic acid was liberated from aerobic cultures over a 24 day incubation period. Although mineralization of *trans*-4-pentylcyclohexanecarboxylic acid was not measured in our study, HPLC analyses (Figure 2.4A) clearly showed that it is susceptible to biodegradation within 20 days of incubation, consistent with the findings of Herman et al. (1994).

Naphthenic acids extracted from MLSBF were also used in biodegradation studies reported by Herman et al. (1994). From their GC analyses data, about 25% of the naphthenic acids were removed from their cultures after 24 days of incubation. The

results shown in Figure 2.4C show about a 40% decrease in the concentration of MLSBF naphthenic acids in our studies. Different MLSBF naphthenic acids preparations and different microbial cultures were used in these two studies. Nonetheless, both studies demonstrated that the naphthenic acids in the Mildred Lake Settling Basin are susceptible to biodegradation.

The data in Figure 2.5 unequivocally demonstrate that mineralization accompanies the biodegradation of the Kodak naphthenic acids. Herman et al. (1994) worked with a similar preparation, the Kodak salts, and observed 47% to 68% of the organic carbon converted to inorganic carbon over a 25 day incubation. In contrast, the cultures used for my mineralization experiment showed only about 14% mineralization over a 40 day period.

I am not aware of any previous reports on the biodegradation of the Merichem naphthenic acids. However, the data in Figure 2.4B show that the Merichem naphthenic acids are also susceptible to biodegradation, and the HPLC method showed about a 30% decrease in the concentrations of these acids. Thus, each of the naphthenic acids preparation that I tested was biodegraded to some extent.

Figure 2.4A indicates that most of the *trans*-4-pentylcyclohexanecarboxylic acid was degraded by viable mixed cultures by the end of the 30 day incubation period. In contrast, more than half of the Merichem and MLSBF naphthenic acids remained in the respective cultures at the end of their incubation periods (Figures 2.4B and 2.4C). These results are somewhat predictable because of the different types of compounds in the cultures. The mixed culture that contained *trans*-4-pentylcyclohexanecarboxylic acid had only one compound to degrade, and its structure is quite simple (Chapter 1, Figure 1.1). Once the culture became adapted to degrading this compound, it would be expected to consume all of the *trans*-4-pentylcyclohexanecarboxylic acid. On the other hand, those cultures that contained the naphthenic acids were subjected to a wide variety of compounds to degrade, including those with complex structures as shown in Chapter 1, Figure 1.1. Microorganisms with the ability to degrade some of the compounds in the naphthenic acids mixture would not necessarily be able to degrade all of the compounds therein. Thus, it is very likely that the more persistent compounds would remain in the cultures that contained the naphthenic acids, as was observed in Figures 2.4B and 2.4C

where not all naphthenic acids were biodegraded. The presence of naphthenic acids in petroleums and the oil sands ore indicate that they persist for long periods of time in nature.

Each panel in Figure 2.4 contains about 30 data points, resulting from the analyses of all the sterile controls and the viable cultures sampled during the course of the experiment. The analyses of the samples for each panel were done in a single day. The personnel time required for the preparation and derivatization of 30 samples was about 5 h. Then the derivatized samples were loaded in the autosampler, and the HPLC operated (unattended) for about 6 h to complete all of the analyses. Currently, the most commonly used method for the quantification of naphthenic acids in aqueous samples involves extractions with dichloromethane, further sample preparation, and FT-IR analysis (Jivraj 1995, Holowenko et al. 2001). The analysis of 30 samples using the FT-IR method requires about 3 days (B. Fung, Syncrude Research Centre, personal communication).

Like the FT-IR method, the GC methods (Herman et al. 1994, Jones et al. 2001) for quantifying naphthenic acids in laboratory cultures require the extraction of the acids from the aqueous medium. These extractions are time consuming and they require fairly large sample volumes to extract – likely from 10 to 100 mL of culture, depending on the concentration of naphthenic acids. In contrast, although I usually removed and saved a 3 mL sample for HPLC analysis, only 100 μL of centrifuged culture supernatant is actually used in the reaction mixtures. Thus, small-volume cultures can be prepared and monitored. This saves on the amount of culture medium required, and reduces the shaker and incubation space needed for these biodegradation studies.

Clearly, the HPLC method saves a considerable amount of preparation and analysis time compared to the other available methods for quantifying naphthenic acids. Unfortunately, the HPLC method (as it now exists) exhibits day-to-day variability. Work should be continued to find the source of this problem, and eliminate the day-to-day variability. Nonetheless, the HPLC method has been proven to be a convenient method for measuring the biodegradation of naphthenic acids in laboratory cultures. The simplicity of the method allows more frequent monitoring of the biodegradation of naphthenic acids in laboratory cultures than has been reported previously by other investigators.

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3. A STATISTICAL COMPARISON OF NAPHTHENIC ACIDS CHARACTERIZED BY GC-MS[♣]

3.1 Introduction

Naphthenic acids are found in many petroleums (Lochte and Littman 1955, Fan 1991, Brient et al. 1995, Slavcheva et al. 1999, Jones et al. 2001). These relatively low molecular weight components (typically <500 Da) are readily dissolved as their carboxylates in water at neutral and alkaline pH. They are a complex mixture of alkyl-substituted acyclic and cycloaliphatic carboxylic acids with the general chemical formula $C_nH_{2n+Z}O_2$, where n indicates the carbon number and Z is zero or a negative, even number that specifies a homologous series. Chapter 1, Figure 1.1 shows examples of typical structures of naphthenic acids. The ring structures predominantly contain 5- or 6-carbon atoms in various combinations. Naphthenic acids with one ring belong to the Z=-2 family, those with two rings belong to the Z=-4 family and so on. Naphthenic acids in the Z=0 family are acyclic, but they are more likely to be branched, rather than linear natural fatty acids. For example, Brient et al. (1995) reported that C14 to C20 acyclic isoprenoid acids have been isolated from California crude petroleum.

Brient et al. (1995) summarized the commercial uses of naphthenic acids. Over two-thirds of the naphthenic acids that are produced are converted to metal salts with the largest amount made into copper naphthenate, which is used as a wood preservative. Other metal naphthenates are used as paint driers, lubricants, and fuel additives. Cobalt naphthenate is used in tire manufacture (Brient et al. 1995).

Naphthenic acids cause corrosion during oil refining (Slavcheva et al. 1999) and they may be present in wastewaters at petroleum refineries (Wong et al. 1996). These acids are abundant in the process-affected waters at the oil sands extraction plants in Canada (Schramm et al. 2000, Holowenko et al. 2002, Rogers et al. 2002a). The naphthenic acids are naturally-occurring surfactants and they have been shown to play a

[♣] A version of this chapter has been published. J.S. Clemente, N.G.N. Prasad, M.D. MacKinnon, and P.M. Fedorak. 2003. Chemosphere. 50: 1265-1274.

role in bitumen extraction in the oil sands industry (FTFC 1995, Schramm et al. 2000). The Athabasca Basin in northeastern Alberta, Canada is one of the largest reserves of hydrocarbons in the world, estimated to contain over 1.7 trillion barrels of bitumen (MacLean 1998). The oil sands industry is producing over 120 million barrels of a light sweet crude oil annually, but within the next decade this is expected to increase to as much as 400 million barrels per year.

Most of the present extraction of bitumen from oil sands is based on the Clark caustic hot water extraction process, in which the oil sand ore is digested with warm water (50 °C to 80 °C) and a conditioning agent (NaOH) (Schramm et al. 2000). The bitumen is separated from the sand as a froth and refined. For each m^3 of oil sand processed, about 3 m^3 of water are required and this means that about 4 m^3 of fluid tailings are produced. The extraction tailings slurry consists mainly of solids (sand and clays), water, dissolved organic and inorganic compounds, and un-recovered bitumen (MacKinnon 1989, Mikula et al. 1996). The oil sands companies do not release any extraction wastes from their property leases, so that the process-affected waters and fluid tailings are contained on site, primarily in large settling ponds.

Naphthenic acids are released from the bitumen during digestion with water, particularly under the elevated pH during oil sands processing (Schramm et al. 2000, MacKinnon and Boerger 1986). Resulting process-affected waters have been shown to have naphthenic acids concentrations in the range of 40 to 120 mg/L (Holowenko et al. 2000, 2001, Schramm et al. 2000).

Ultimately, the huge tailings containment areas holding process-affected waters that are being formed during the oil sands operations must be reclaimed into sustainable ecosystems. One of the major challenges is to address the toxicity issues associated with the naphthenic acids. Several studies have demonstrated their toxicity to different organisms (Dokholyan and Magomedov 1983, MacKinnon and Boerger 1986, FTFC 1995, Rogers et al. 2002b). It has been demonstrated that natural aging of oil sands tailings water reduces its toxicity (MacKinnon and Boerger 1986, Holowenko et al. 2002) and that microbial activity in laboratory cultures also reduces the toxicity of naphthenic acids (Herman et al. 1994). However, because of the complexity of the mixture of

compounds that comprise the naphthenic acids, it has not been possible to determine which compounds are responsible for the toxicity.

Holowenko et al. (2002) used the derivatization method of St. John et al. (1998) and gas chromatography-electron impact mass spectrometry (GC-MS) to characterize naphthenic acids in process-affected waters from the oil sands industry. Holowenko et al. (2002) presented their data in three-dimensional plots as illustrated in Figure 3.1. They observed that a "valley" was often evident at carbon numbers 19 to 21. In this analysis, the group of naphthenic acids with carbon numbers 22 to 33, in Z families 0 to -12 was defined as the C22+ cluster. Holowenko et al. (2002) demonstrated that as the proportion of naphthenic acids in the C22+ cluster increased, the acute toxicity (measured by the Microtox™ bioassay method) decreased.

Visual inspection of the three-dimensional plots such as those shown in Figure 3.1 suggests that the compositions of the two mixtures of naphthenic acids are different. However, we sought a statistical method to compare naphthenic acids from different sources. The method, based on the t-test, allowed the comparison of naphthenic acids from a variety of sources, including oil sands process-affected waters, commercial preparations, and oil sands ores.

3.2 Materials and Methods

3.2.1 Naphthenic acids

Four commercially available preparations of naphthenic acids were used in this study. Naphthenic acids sodium salt (P9513) and naphthenic acids (P2388) were purchased from The Eastman Kodak Company (Rochester, NY). Crude and refined naphthenic acids were a gift from Merichem Chemicals and Refinery Services LLC (Houston, TX).

Naphthenic acids were extracted into dichloromethane from process-affected waters from Syncrude Canada Ltd., as outlined by Holowenko et al. (2002). Two extracts, designated MLSBF and Pit 5, are discussed in this chapter. The former represents fresh extraction tailings taken from the surface water zone of the Mildred Lake

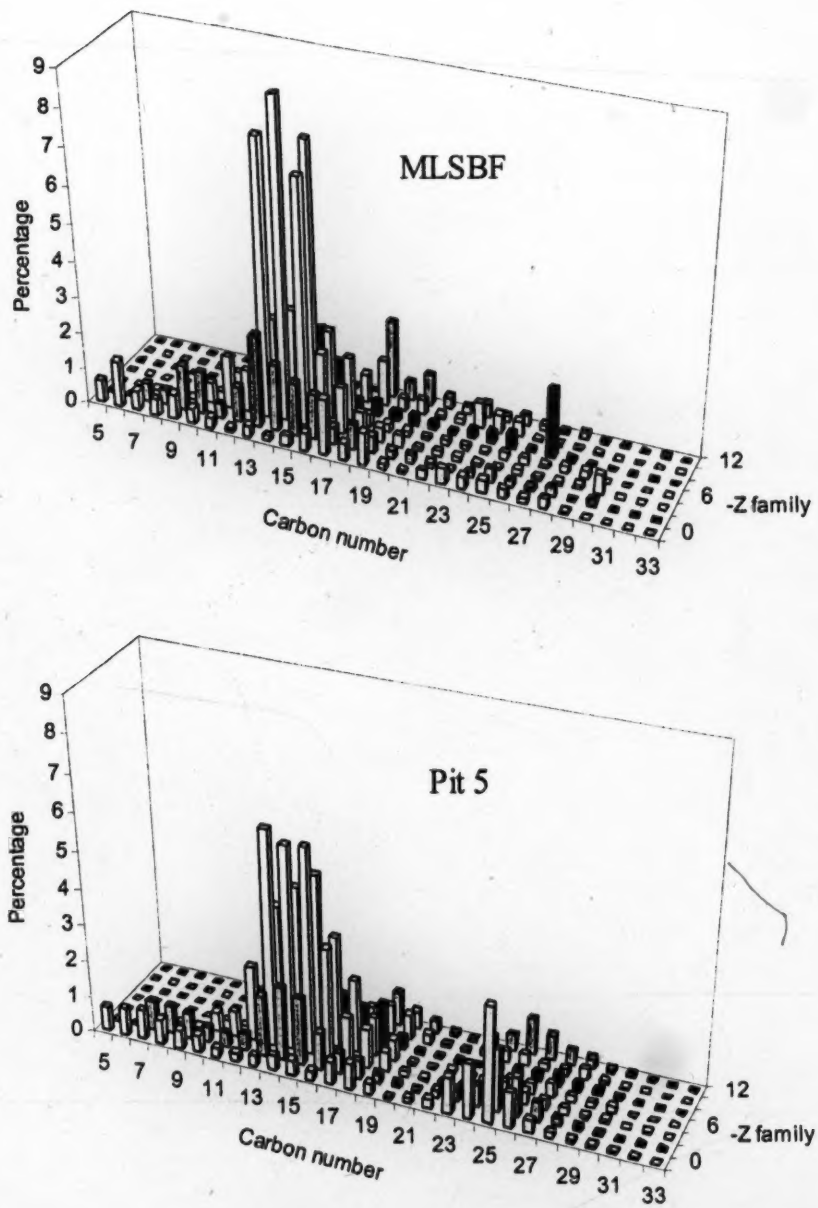

Figure 3.1 The distribution of ions corresponding to various carbon numbers and Z families in the complex naphthenic acids mixtures from MLSBF and Pit 5 samples from Syncrude. The bars represent the percentage (by number of ions) of naphthenic acids in the mixture that account for a given carbon number in a given Z family (corresponding to specific m/z values from GC-MS analysis). The sum of all the bars equals 100%.

Settling Basin. The latter sample was water from Mildred Lake Settling Basin that had acted as a capping layer for mature fine tailings in a storage pit designated Pit 5. This was isolated from fresh input of process waters and it had aged under natural conditions for 11 years.

Naphthenic acids were also extracted from two oil sands ore samples. One came from the Aurora mine site on the Syncrude lease, and the other originated from the mining operation of Suncor Energy Inc. The method for extracting naphthenic acids from the ore is given by Holowenko et al. (2002). Briefly, the ore sample was digested with 1 M NaOH, and after centrifugation to remove solids, the aqueous phase was acidified to pH 2 to 2.5 with H_2SO_4 and the naphthenic acids were extracted into dichloromethane. The concentrations of naphthenic acids in the dichloromethane extracts were determined by Fourier-transform infrared spectroscopy (Jivraj et al. 1995, Holowenko et al. 2001).

3.2.2 Derivatization and GC-MS

Naphthenic acids were derivatized as outlined by St. John et al. (1998) using *N*-methyl-*N*-(*t*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) which contained 1% *t*-butyldimethylsilylchloride (Sigma, St. Louis MO). The GC-MS method was described by Holowenko et al. (2002).

Spectral data were acquired using the Mass Spec Data System for Windows version 14.0c (Mass Spec Services, England). Peak ion intensity values were averaged over the elution of the naphthenic acids hump, generally from retention time 10 min onward. The "minimum occurrence" variable for the averaged data was set at 1%, which meant that the peak ion had to occur in at least 1% of the total scans averaged to be included in the final average data outputted from the computer. The averaged peak intensity values were inputted into a Microsoft Excel spreadsheet which selected only those masses that corresponded to derivatized naphthenic acids with carbon numbers of 5 to 33 and Z values of 0 to -12, filling a matrix as in Figure 3.2. The rows and columns were summed and the total peak intensity value was obtained. The intensity value for each ion was divided by the sum of all the ion intensities to produce a normalized value as shown in Figure 3.2. These normalized data were used to plot three-dimensional

graphs (e.g. Figure 3.1), and to compare the different samples by a t-test. The normalized data provide no information on the total number of peaks detected by GC-MS in the sample, nor any information about absolute concentrations of the various components.

3.2.3 Statistical method

The data in each matrix were divided into three groups (Figure 3.2). Group 1 contained the intensities for all of the naphthenic acids with carbon numbers of 5 to 13, inclusive. Group 2 contained those with carbon numbers of 14 to 21, inclusive, and group 3 included those with carbon numbers of 22 to 33, inclusive. The members of group 3 were chosen to match the C22+ cluster that was observed by Holowenko et al. (2002). Examination of many three-dimensional plots of naphthenic acids preparations suggested that compounds with 13 carbons or less were very abundant in some preparations, whereas those with 14 carbons or more were very abundant in other preparations. Thus, the division between groups 1 and 2 was set between carbon numbers 13 and 14.

Each percent value in the matrix (Figure 3.2) was divided by 100, and the arcsine of each of the quotient was taken as a variance stabilizing transformation. The arcsine-transformed data for each group from one naphthenic acid sample was compared with the corresponding arcsine-transformed data of the corresponding group from a second naphthenic acid sample by applying an independent two-sample t-test assuming equal variance. The groups were considered to be different if the P value for the two-tailed test was <0.05.

3.3 Results and Discussion

The advantage to analyzing the *t*-butyldimethylsilyl derivatives of the naphthenic acids is that little fragmentation occurs in the mass spectrometer (St. John et al. 1998). Typically, the derivatized acid loses $C(CH_3)_3$ to yield a base peak with m/z of 57 mass units greater than the underivatized naphthenic acid. The data collected and summarized in a matrix as shown in Figure 3.2 contains the relative abundance of up to 156 base

MLSBF

C number	Z family 0	-2	-4	-6	-8	-10	-12	% by C no.
5	0.62							0.62
6	1.26							1.26
7	0.56	0.42						0.98
8	0.44	0.40						0.84
9	0.71	1.20						1.92
10	0.44	1.13	0.56					2.13
11	0.30	0.39	1.46					2.15
12	0.13	0.99	1.19	1.58				3.88
13	0.30	2.56	7.49	2.49				12.85
14	0.17	1.89	8.61	2.53	1.17			14.66
15	0.29	1.48	6.74	7.36	2.15			18.03
16	0.51	1.38	2.16	2.49	1.31	0.71		8.46
17	1.59	0.47	1.29	1.78	0.35	0.79		6.25
18	0.46	0.88	0.73	0.37	0.43	1.28	2.11	6.07
19	0.87	0.49	0.43	0.22	0.25	0.36	0.50	3.12
20	0.18	0.21	0.32	0.16	0.29	0.42	0.86	2.44
21	0.11	0.23	0.11	0.10	0.18	0.12	0.33	1.18
22	0.24	0.14	0.14	0.13	0.07	0.14	0.18	1.04
23	0.44	0.09	0.12	0.11	0.24	0.72	0.38	2.10
24	0.33	0.14	0.08	0.07	0.30	0.52	0.18	1.62
25	0.38	0.37	0.09	0.18	0.40	0.44	0.32	2.18
26	0.25	0.17	0.17	0.18	0.07	0.05	0.16	1.06
27	0.17	0.14	0.07	0.11	1.93	0.07	0.12	2.63
28	0.28	0.24	0.08	0.20	0.08	0.05	0.19	1.13
29	0.05	0.00	0.14	0.21	0.06	0.05	0.07	0.57
30	0.00	0.18	0.56	0.00	0.00	0.00	0.05	0.79
31	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
32	0.00	0.00	0.00	0.00	0.00	0.00	0.05	0.05
33	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
% by Z no.	11.09	15.29	32.53	20.54	9.30	5.73	5.52	100.00

Pit 5

C number	Z family 0	-2	-4	-6	-8	-10	-12	% by C no.
5	0.68							0.68
6	0.74							0.74
7	0.84	0.76						1.60
8	0.69	0.73						1.42
9	0.52	0.69						1.15
10	0.47	0.38	0.54					1.44
11	0.26	0.39	0.70					1.35
12	0.23	0.47	2.07	1.09				3.86
13	0.29	1.68	5.79	3.52				11.28
14	0.42	2.02	5.47	4.19	0.72			12.83
15	0.43	1.85	5.85	4.56	1.26			13.75
16	0.27	1.01	3.04	3.09	1.18	0.75		9.33
17	0.84	0.56	1.27	1.93	0.94	0.67		6.00
18	0.72	0.48	1.07	1.08	1.16	0.83	0.92	6.27
19	0.26	0.25	0.54	0.57	0.36	0.68	0.49	3.15
20	0.07	0.12	0.13	0.08	0.08	0.25	0.32	1.05
21	0.16	0.06	0.10	0.05	0.05	0.10	0.12	0.63
22	0.27	0.18	0.10	0.12	0.06	0.07	0.06	0.87
23	0.99	0.36	0.14	0.08	0.06	0.18	0.27	2.08
24	1.50	0.59	0.39	0.19	0.12	0.30	0.45	3.55
25	3.19	1.72	0.51	0.29	0.19	0.32	1.02	7.24
26	1.00	1.10	0.36	0.27	0.25	0.18	0.68	3.86
27	0.35	0.51	0.28	0.42	0.31	0.18	0.36	2.40
28	0.18	0.21	0.17	0.20	0.22	0.31	0.28	1.57
29	0.11	0.13	0.10	0.13	0.13	0.10	0.11	0.81
30	0.10	0.07	0.14	0.16	0.08	0.05	0.11	0.69
31	0.06	0.02	0.03	0.01	0.03	0.03	0.04	0.24
32	0.02	0.03	0.05	0.00	0.00	0.00	0.02	0.13
33	0.00	0.03	0.00	0.00	0.00	0.00	0.00	0.03
% by Z no.	15.46	16.51	25.66	21.93	7.19	4.99	5.26	100.00

t-test results (two-sided test)*			Proportions of ions in each Group	
Comparing samples	MLSBF and Pit 5		MLSBF	Pit 5
Group 1 (C5 to C13)	P= 0.7457	Sums=	26.63 Percent	25.53 Percent
Group 2 (C14 to C21)	P= 0.6498	Sums=	60.21 Percent	53.00 Percent
Group 3 (C22 to C33)	P= 0.0307	Sums=	13.17 Percent	23.46 Percent

*Significant difference if P<0.05

Figure 3.2 Matrices summarizing the percent (by number of ions) given by the formula $C_nH_{2n+Z}O_2$ distributed among carbon numbers and Z families in the MLSBF and Pit 5 samples. The lower portion shows the results from the t-test comparing the three groups in each sample, and the sums of the abundance of the ions in each group. The blank portion represents cases in which there are insufficient numbers of carbon or hydrogen atoms available to form a naphthenic acid with an alkyl substituent and the formula $C_nH_{2n+Z}O_2$. See text for details.

peaks. Twenty-two of the base peaks make up group 1 of Figure 3.2, 50 of the peaks make up group 2, and 84 of the peaks make up group 3. The blank portion of each matrix in Figure 3.2 represents cases in which there are insufficient numbers of carbon or hydrogen atoms available to form a naphthenic acid with an alkyl substituent and the formula $C_nH_{2n+Z}O_2$. For example, the empirical formula for carbon number 6, Z=-12 is C_6O_2, which is devoid of hydrogen and clearly does not fit the formula $C_nH_{2n+Z}O_2$. Holowenko et al. (2002) provide a more complete discussion of assumptions of naphthenic acid structures that exclude entries in the blank portion of the matrix.

Figure 3.1 shows the distribution of ions in the naphthenic acids from the tailings water samples designated MLSBF and Pit 5. These three-dimensional graphs are the result of plotting the data in Figure 3.2, and were presented by Holowenko et al. (2002) without statistical analysis. The Pit 5 sample (Figure 3.1) clearly shows the "valley" at carbon number 21. This "valley" was observed in other oil sands process-affected waters examined by Holowenko et al. (2002), leading to the designation of the C22+ cluster in that publication, and to the delineation of group 3 in this study. The t-test results for group 3 in these two samples showed that there was a significant difference (P=0.031, Figure 3.2) between them. About 13% of the ions detected in the MLSBF sample were in group 3, whereas about 23% of the ions in the Pit 5 sample were in group 3 (Figure 3.2). The remainder of the ions were in groups 1 and 2. Holowenko et al. (2002) hypothesized that biodegradation of the lower molecular weight naphthenic acids would lead to a decrease in the proportion of ions found in the C<22 cluster (i.e. groups 1 and 2), with a corresponding increase in the relative abundance in the C22+ cluster. The sums in Figure 3.2 show that the proportions of ions found in groups 1 and 2 of the Pit 5 sample were smaller than in the MLSBF sample, but the t-tests found no significant differences between these individual groups in the two samples.

The data presented in the remainder of this chapter are the means from triplicate GC-MS analyses of various derivatized naphthenic acids preparations. In all but one case, when the results of the replicate analyses of a given naphthenic acids preparation were compared using the t-test method, no differences between replicate analyses were detected. This indicated good reproducibility between GC-MS analyses. The only

exception was that one of the three replicate analyses of the crude Merichem preparation showed a significantly higher group 3 content than the other two replicate analyses.

Several biodegradation studies have used commercially available naphthenic acids as surrogates for naphthenic acids found in oil sands wastewaters (Herman et al. 1994, Lai et al. 1996, Holowenko et al. 2001). Two of these are the naphthenic acids and the sodium salts of naphthenic acids that were available from Kodak. The distributions of ions in these commercial preparations are shown in Figure 3.3. The proportions of ions found in groups 1, 2 and 3 in the Kodak acids were approximately 70%, 30% and 0%, respectively. In contrast, the proportions of ions found in groups 1, 2 and 3 in the Kodak salts were approximately 21%, 79% and 0.3%, respectively. Comparing groups 1 to 3 in these two preparations by t-tests, gave P values of 0.018, 0.011, and 0.041, respectively. Thus, each of the three groups are significantly different in the two Kodak preparations.

There is a marked difference in the appearances of the Kodak preparations (Figure 3.3) and the naphthenic acids from the MLSBF (MLSBF, Figure 3.1). Most notably, the Kodak preparations are nearly devoid of naphthenic acids that fall into the C22+ cluster (group 3), and the commercial preparations have a few rather abundant ions. For example, the Kodak acids (Figure 3.3) have two ions that comprise 12% and 14% of the naphthenic acids, whereas the most abundant ion in the MLSBF sample (Figure 3.1) comprises only 9% of the naphthenic acids.

Comparing the MLSBF sample with the Kodak acids by the t-tests gave P values of 0.045, 0.066 and <0.001 for groups 1, 2 and 3, respectively. Thus, two of the three groups in the two preparations differed significantly from each other. In contrast, the t-test comparison of the MLSBF sample and the Kodak salts gave P values of 0.472, 0.357 and <0.001. Based on the results from comparisons of groups 1 and 2, the Kodak salts are quite comparable to the MLSBF sample. These statistical analyses suggest that the Kodak salts preparation would be the better surrogate for the naphthenic acids found in the MLSBF. However, this conclusion is based solely on the distribution of ions in the naphthenic acids samples, and not on the actual chemical structures of the compounds in the samples. To date, there is no information on the exact structures that make up the ions for a given carbon number and Z value.

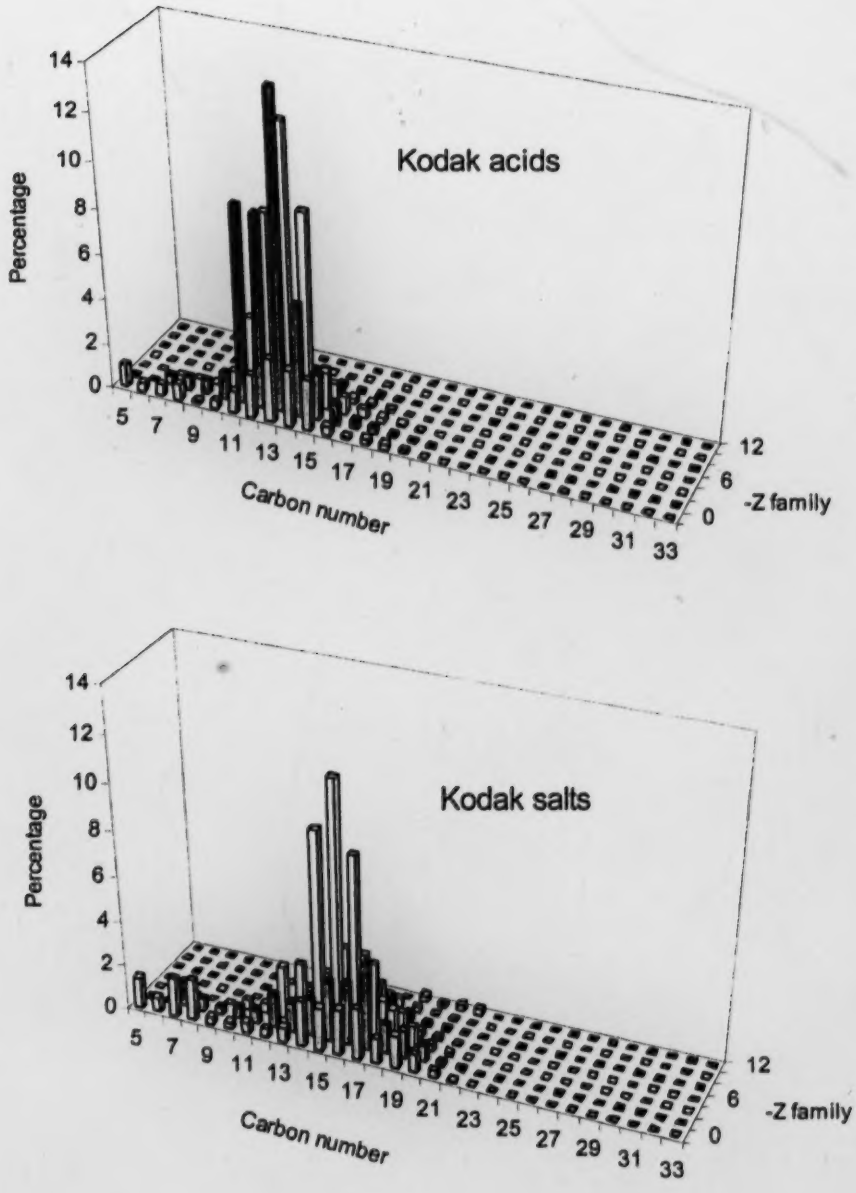

Figure 3.3 The distribution of ions corresponding to various carbon numbers and Z families in the commercial preparations of Kodak naphthenic acids and Kodak naphthenic acids sodium salts. The bars represent the percentage (by number of ions) of naphthenic acids in the mixture that account for a given carbon number in a given Z family (corresponding to specific m/z values from GC-MS analysis). The sum of all the bars equals 100%.

Two grades of naphthenic acids were obtained from Merichem. One was a crude product with a dark brown color. The crude preparations typically have acid number between 160 and 230, and unsaponifiables (usually hydrocarbons and phenols) of 7% to 30% by weight (Merichem 2002). The refined product had an acid number of 257, and was a clear, golden color. The refined preparations typically contain unsaponifiables of 4% to 8% by weight (Merichem 2002). The two Merichem preparations were only distinguishable on the basis of group 3 (P=0.003), with no detectable ions in this group in the refined preparation and 0.78% of the detected ions in this group in the crude product. Like the Kodak preparations (Figure 3.3), the Merichem products were essentially devoid of compounds in the C22+ cluster (group 3). The t-test results showed that the refined Merichem preparation was markedly different than the Kodak salts, with P values of 0.003, 0.003 and 0.041 for groups 1, 2 and 3, respectively.

The naphthenic acids in the extraction tailings water that reach the settling ponds in the oil sands operations originate from the ores and are released during the aqueous digestion used in the extraction process. The ores are derived from various depths and locations, and they originated from various depositional and post-depositional environments. Natural processes of groundwater and microbial actions on the bitumen and its constituents can be expected. This means that the ores that are mined from different locations could have naphthenic acid groupings that reflect *in situ* changes on the concentrations and composition of the naphthenic acids present. Thus, it is likely that different ores contain different compositions of naphthenic acids.

Figure 3.4 compares the naphthenic acids extracted from two different ore samples. The mining sites for Syncrude's Aurora ore and the Suncor (86 site) ore are about 30 km apart. The naphthenic acid content in the Syncrude sample was 150 mg/kg of oil sands, and that in the Suncor was 370 mg/kg of oil sands. Nearly 23% of the ions detected in the Suncor sample were in the C22+ cluster (group 3), compared to only about 6% in the Syncrude sample. The P values from the t-tests on groups 1, 2 and 3 were 0.015, 0.049 and <0.001, respectively. Thus, all three groups were significantly different in these two ore samples. Group 1 comprised approximately 16% of the detected ions in the Syncrude sample and 26% in the Suncor sample.

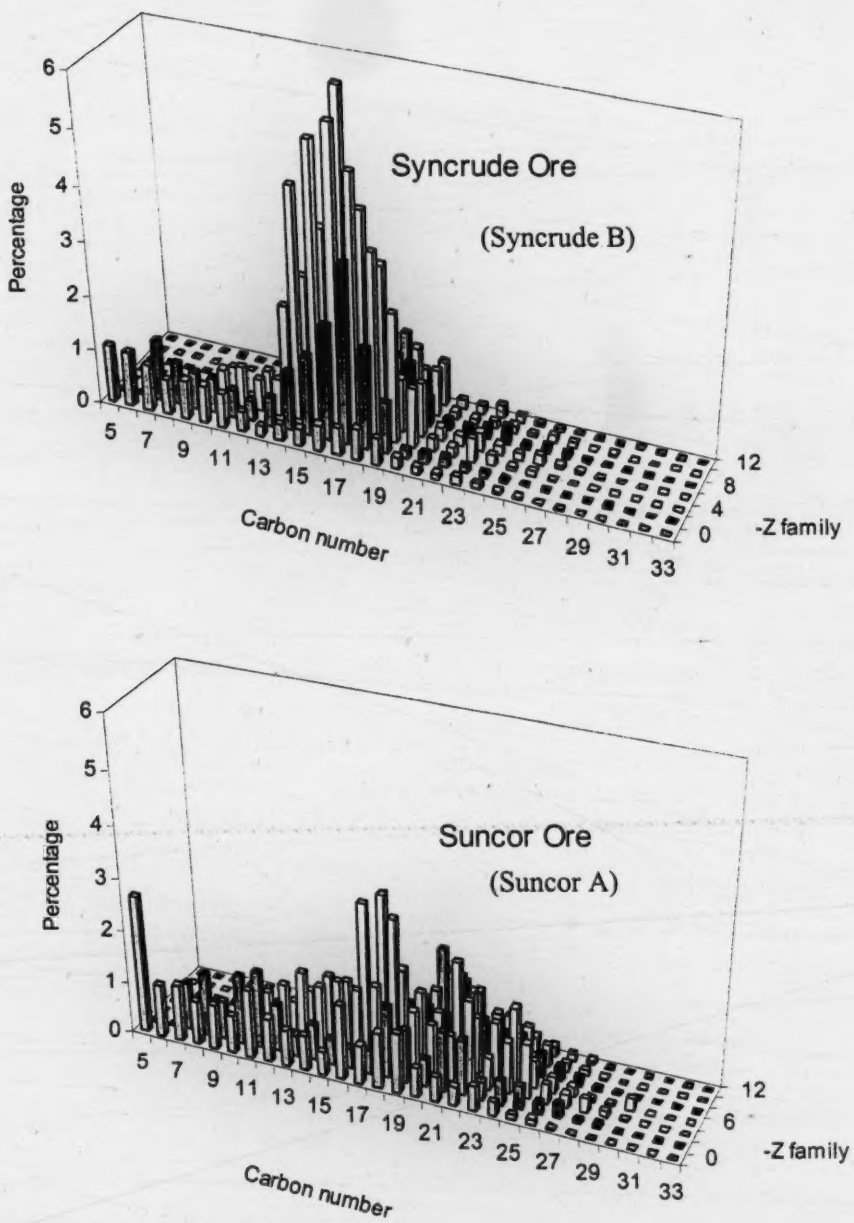

Figure 3.4 The distribution of ions corresponding to various carbon numbers and Z families in the naphthenic acids extracted from oil sands ore samples from Syncrude and Suncor. The bars represent the percentage (by number of ions) of naphthenic acids in the mixture that account for a given carbon number in a given Z family (corresponding to specific m/z values from GC-MS analysis). Labels in parenthesis are the same as in Chapter 4 and the Appendix A.B. The sum of all the bars equals 100%.

St. John et al. (1998) used this GC-MS method to distinguish between two naphthenic acid preparations from different suppliers. The two preparations were vastly different and were differentiated by the percent by Z number (the bottom row of the matrix in Figure 3.2). For example, the Z=0 family in one preparation comprised 6.7% of the naphthenic acids, whereas the same family in the second preparation comprised 58% of the naphthenic acids (St. John et al. 1998). Examining the "% by Z no." values in the two matrices in Figure 3.2 indicates that it would be difficult to distinguish between the MLSBF and Pit 5 samples, because there are not large differences between any two corresponding values. For example, for the Z=0 families, the "% by Z no." values for the MLSBF and Pit 5 samples are 11.09% and 15.46%, respectively. Holowenko et al. (2001) used the same approach to differentiate between the Kodak salts and a sample of naphthenic acids obtained from the MLSBF. The method presented in this chapter allows a statistical comparison of naphthenic acids from various sources, and provides information on which of the three groups (based on carbon numbers) are statistically different from each other. This method proved to be useful for distinguishing among commercial preparations and samples derived from the oil sands operations.

Although the focus of this chapter is on the application of the t-test method to data collected from GC-MS analyses, the method can be applied to results collected by other mass spectrometry procedures used to characterize naphthenic acids, such as chemical ion ionization mass spectrometry (Fan 1991, Hsu et al. 2000), fast atom bombardment mass spectrometry (Fan 1991, Wong et al. 1996) or electrospray ionization mass spectrometry (Morales-Izquierdo 1999). Regardless of the procedure used, once the data are summarized in the matrices as illustrated in Figure 3.2, the t-test method can be applied to compare the distribution of ions in the naphthenic acids.

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4. COMPARISON OF NAPHTHENIC ACIDS DISTRIBUTION IN OIL SANDS ORES

4.1 Introduction

Crude oils naturally contain naphthenic acids (Seifert and Teeter 1969, Seifert et al. 1969, Fan 1991, Tomczyk et al. 2001). They are found at concentrations of 0.0015% in Egyptian crude and 2% (w/w) in the Athabasca oil sands (CONRAD 1998). The carboxylic acids found in crude oil deposits are a result of having immature deposits or biodegraded mature deposits (Tissot and Welte 1978). Athabasca oil sands naphthenic acids come from the biodegradation of mature petroleum (Tissot and Welte 1978). Naphthenic acids are a chemically and structurally related group of compounds defined by the general formula $C_nH_{2n+Z}O_2$, where n represents the carbon number and Z is related to the number of rings. Chapter 1, Figure 1.1 shows possible naphthenic acid structures. They may be cyclic ($Z<0$) or acyclic ($Z=0$). Cyclic compounds also contain an alkyl substituent. The acyclic components of naphthenic acids, unlike fatty acids, are highly branched (Rudzinski et al. 2002). The smallest cyclic structure expected in a naphthenic acids mixture is a cyclopentanecarboxylic acid, while the largest compound is expected to be <1000 Da (CONRAD 1998).

The presence of naphthenic acids in crude oil has been related to operational problems. They are implicated in crude oil processing equipment corrosion. This is found to occur when temperatures are between 220 °C to 400 °C, in locations experiencing high velocity (Kane and Cayard 1999, Turnbull et al. 1998). Not only the concentration, but the structure of compounds in the naphthenic acids mixture also plays a role in corrosion (Turnbull et al. 1998).

Petroleum from the Athabasca oil sands is extracted using the Clark caustic extraction method. This involves exposing the oil sands to steam, and sodium hydroxide to separate bitumen from the sand (Schramm et al. 2000). The resulting wastewater, referred to as tailings, is stored in settling ponds such as the Mildred Lake Settling Basin, which was created by Syncrude in 1978 (MacKinnon 1989). This artificial lake has a

volume of 300 x 10^6 m^3 (Leung et al. 2001). Naphthenic acids are believed to be among the most toxic component of oil process wastewaters (Madill et al. 2001) and petroleum refinery wastewaters (Wong et al. 1996). They are toxic to plants (Kamaluddin and Zwiazek 2002), mammals (Khanna et al. 1971, Rogers et al. 2002), and fish (Dokholyan and Magomedov 1981). However, this toxicity decreases with naphthenic acids biodegradation (MacKinnon and Boerger 1986, Herman et al. 1994, Holowenko et al. 2002).

The composition of naphthenic acids mixtures is believed to play a role in the observed toxicity. In particular, emergence of compounds with greater than 22 carbons, labeled the C22+ cluster, coincides with decreasing naphthenic acids concentrations and toxicity (Holowenko et al. 2002). Such studies are made possible using mass spectrometry. The observed masses can be displayed in a two-dimensional matrix, using carbon number and Z-family combinations to determine expected masses of compounds, as shown by St. John et al. (1998). Holowenko et al. (2002) further restricted the masses considered to only those that follow the $C_nH_{2n+Z}O_2$ rule, where rings also have alkyl substituents. Although visual inspection of the three-dimensional graphs allows the differentiation of naphthenic acids mixtures from different sources, a statistical comparison of the ions has been applied. An independent samples t-test after arcsine transformation was used to determine differences between two naphthenic acids mixtures (Chapter 3). It was found that there are statistically significant differences between two commercial naphthenic acids (Kodak salts and Merichem acids), and two oil sands ores (Syncrude and Suncor).

The carboxylic acid fraction of petroleum has been used as an indicator of the extent of biodegradation the deposit has experienced. The ratio of tricyclic to pentacyclic terpanoic acids for example, helped determine the extent of biodegradation, as well as the events that have contributed to the deposition of petroleum in the Albacora oil field in Brazil (Nascimento et al. 1999). Determining differences in naphthenic acids composition may also provide clues to the different sources of the naphthenic acids, and their source petroleum. Barrow et al. (2003) compared the naphthenic acids fraction from two oil fields. They found that although the naphthenic acids distribution fell within similar m/z values, the abundance of individual compounds from each oil field differed.

Holowenko et al. (2002) found that Syncrude and Suncor wastewaters had different levels of toxicity, total naphthenic acids concentrations, and naphthenic acids component distributions. Since the oil sands ores are the sources of these naphthenic acids, determining whether the location of the ore deposit within the Athabasca oil sands is a contributing factor in the observed naphthenic acids distribution is essential. Confounding factors, such as differences in the methods used in ore extraction and wastewater management may have also contributed to the observed differences found by Holowenko et al. (2002). A statistical method, applied here, which allows for comparisons among ore samples may illuminate the source of variation in naphthenic acids compound distribution. Naphthenic acids extracted from oil sands ores were analyzed using the method developed by St. John et al. (1998), as applied by Holowenko et al. (2002). The resulting ion abundances were then compared after logit transformation and dividing the data into three groups based on carbon number.

4.2 Materials and Methods

4.2.1 Oil sands ore samples

Syncrude, True North, Suncor, and Albian provided oil sands ore samples (Table 4.1). Naphthenic acids from oil sands ores were extracted by personnel at the Syncrude Research Centre, as described by Holowenko et al. (2002), using 1 M NaOH followed by centrifugation. The supernatant, containing naphthenic acids, was acidified to pH 2.0 using H_2SO_4 and extracted with CH_2Cl_2. Among the ore samples, True North C, Syncrude F, and Syncrude G underwent extraction twice. The samples represented ores with different ore processing performance (Syncrude A = Aurora basal ore, poor processing; Syncrude B = Aurora basal ore, good processing), and from different leases (Syncrude Aurora, North mine, True North, Suncor, Albian) in the Athabasca deposit north of Fort McMurray, as well as from different sources (depositional environment) within each lease (i.e. Albian A = estuarine, Albian B = fluvial, Albian C = tidal). Concentrations of naphthenic acids in CH_2Cl_2 were determined by Syncrude using Fourier Transform Infrared Spectroscopy (FT-IR), as described by Jivraj et al. (1995).

Table 4.1 Summary of the description, naphthenic acids concentration (mg NA/kg oil sand), and bitumen content (%w/w oil sand) of the Athabasca oil sands ore samples.

Designation	Description	mg NA[a] / kg oil sand	Bitumen content (%w/w oil sand)
Syncrude A	Aurora Basal ore, poor processing	51	12
Syncrude B	Aurora Basal ore, good processing	149	13
Syncrude C	Aurora 1 km N. face F7x	74	14
Syncrude D	Aurora 1 km N. face F8	69	11
Syncrude E	Aurora Transition ore F11	504	10
Syncrude F	North mine Upper bench	317	14
Syncrude G	North mine Lower bench	304	15
True North A	True North #4C	123	12
True North B	True North #5	122	15
True North C	True North #28A	131	12
Suncor A	Suncor Elev. 830	373	14
Suncor B	Suncor Steepbank	396	15
Albian A	Albian Tidal	na[b]	na
Albian B	Albian Estuarine	na	na
Albian C	Albian Fluvial	na	na

[a] Naphthenic acids
[b] data not available

Information on the samples is summarized in Table 4.1. The description of each sample is included, as well as the naphthenic acids concentrations determined using FT-IR. Concentrations for the Albian samples were not available.

4.2.2 Derivatization and GC-MS analysis

Naphthenic acid extracts were derivatized to their *t*-butyldimethylsilyl esters as described by St. John et al. (1998). Samples in CH_2Cl_2, at concentrations between 10 to 30 mg/mL (measured gravimetrically) were derivatized using *N*-methyl-*N*-(*t*-butyl-dimethylsilyl)trifluoroacetamide (MTBSTFA) which contained 1% *t*-butyldimethylsilyl chloride (Sigma, St. Louis MO). GC-MS analyses were performed as outlined by Holowenko et al. (2002). Spectral data were acquired using Mass Spec Data for

Windows version 14.0C (Mass Spec Services, England). The naphthenic acids peaks were averaged from 10 min until the signal returned to baseline; using a 1% minimum occurrence variable (the mass must appear in at least 1% of the total scans to be included in the average). The scans were then transposed into an Excel macro, which restricts the peaks analyzed to those that follow the definition of naphthenic acids as described by Holowenko et al. (2002) and summarizes the data as three-dimensional graphs.

4.2.3 Statistical analysis

Statistical analyses were performed using SPSS for Windows version 11.0. Ions for each sample were divided into three groups as in Chapter 3. Group one contained ions with carbon numbers 5 to 13, group 2 were ions within carbon 14 to 21, and group 3 were ions within carbon 22 to 33. Each group was analyzed separately using linear mixed models, where means were compared between samples using pairwise comparison. No adjustment was made for the fact that multiple comparisons were being employed (least significant difference, equivalent to a t-test according to SPSS 11.0). Prior to analysis, the data expressed as percentages were logit transformed as shown by Equation 4.1:

$$\ln\left[\frac{(\text{percent} / 100) + 0.00001}{1 - ((\text{percent} / 100) + 0.00001)}\right]$$

Equation 4.1

Logit transformation is commonly used to improve the variance and normality of values expressed as proportions (Ramsey and Schafer 1997). Adding a sufficiently small number such as 0.00001 allows the inclusion of 0 values, which would otherwise be ignored. A $P<0.05$ was considered statistically significantly different. Samples from Chapter 3 were also analyzed using this method, these include: Kodak salts, Kodak acids, and Refined Merichem.

The logit transformed data were also analyzed using hierarchical cluster analysis. Squared Euclidean distance and within group average linkage were used. These parameters were chosen because they did not result in any change in groupings when samples were added or subtracted. SPSS 11.0 was utilized for this analysis. Unlike the pairwise comparisons, the data were not divided into three groups prior to analysis.

4.3 Results and Discussion

4.3.1 Effects of transformation on data structure

Logit transformation is used for proportion data (Ramsey and Schafer 1997). This improves the normality and variance of the samples, which is important in parametric statistical analyses. Figure 4.1 shows box plots, diagrams of data distribution, illustrating the effect of transformation on the data structure. The boxes represent the middle 50% of the data, with the median shown by a horizontal line; outliers, data which were more than 1.5 box lengths away are shown as open circles, and extreme outliers, data which were more than 3 box lengths away are shown as asterisks (Ramsey and Schafer 1997). Data shown here are group 2 compounds (as defined in Chapter 3) considered as naphthenic acids, because they follow the $C_nH_{2n+Z}O_2$ rule, where rings also contain alkyl moieties. This group was chosen because it generally contains the majority of the ions.

Box plots for groups 1 and 3 are shown as Appendix A.A. Figure 4.1A is the untransformed data for Kodak salts, Kodak acids, Refined Merichem, and MLSBF. Figure 4.1B shows the data after arcsine transformation (as in Chapter 3); the distributions are similar to those in Figure 4.1A, with the exception that the values along the y-axis are 0.01 times that of the untransformed values. Figure 4.1C shows the data after logit transformation, where values of -11.5 translate to 0% in the untransformed graph. The horizontal line for the Merichem acid logit transformed data (Figure 4.1C) is at -11.5. The Merichem acid three-dimensional graph in Chapter 6 (Figure 6.4) shows that most of its ions are in group 1 (Carbon numbers 5 to 13). Kodak acids ions mostly belong to group 1 as well, but this commercial mixture also contains more group 2 ions

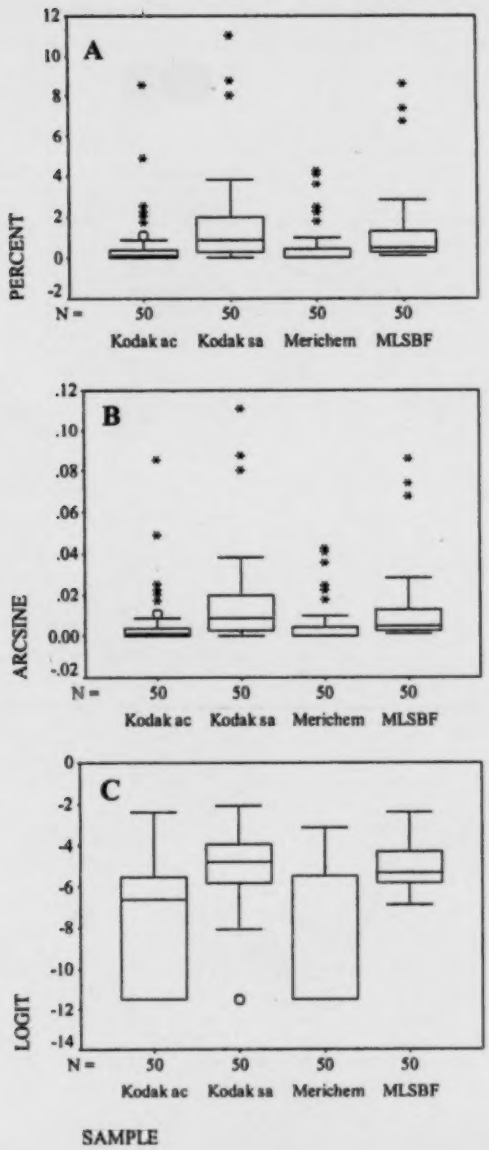

Figure 4.1 Effect of transformation on the data structure of group 2 ions from Kodak acids (Kodak ac), Kodak salts (Kodak sa), Refined Merichem (Merichem), and MLSBF (Chapter 3). Three data manipulations are shown: untransformed (A), arcsine transformed (B), and logit transformed (C). Outliers are represented as open circles, and extreme outliers are shown as asterisks. More detailed descriptions are in the text.

compared to Merichem acids (Chapter 3, Figure 3.3). Kodak acids plot in Figure 4.1C therefore has a higher median compared to Merichem acids. Logit transformation pulls

extremely large values closer to the median and makes them less influential, since this involves taking the natural logarithm of the ion abundance (Figure 4.1C). Figure 4.1C has only one outlier (from Kodak salt), unlike Figures 4.2B and 4.2C where there are a number of extreme outliers for all four data sets. Ions which make up a smaller proportion of the total ions become relatively more important, when compared to the untransformed, and even the arcsine transformed data.

In Chapter 3, Kodak salts, Kodak acids, Refined Merichem, and MLSBF were analyzed using a t-test after arcsine transformation. Table 4.2 is a summary of the results of both the arcsine – t-test (italics) and logit – pairwise (bold) comparisons for these samples. It was necessary to determine whether conclusions derived from both methods agreed with each other. Overall, analysis after logit transformation produced conclusions similar to those from independent samples t-test after arcsine transformation: There were differences in all groups between Kodak salts and Kodak acids; Refined Merichem, and Kodak salts. There were also differences in group 3 of Kodak salts and MLSBF; group 3 of Kodak acids and MLSBF; and groups 2 and 3 of Refined Merichem and MLSBF. However, there were also discrepancies between the two methods. Arcsine – t-test found differences in group 1 after comparing Refined Merichem and MLSBF acids; as well as Kodak acids and MLSBF. Logit – pairwise comparison on the other hand did not detect a significant difference among ions in group 1 after comparing Refined Merichem and MLSBF; a difference in group 2 instead of group 1 was also found after comparing Kodak acids and MLSBF. In addition to that, arcsine – t-test found no statistical difference between Kodak acids and Refined Merichem, while logit – pairwise found differences in the group 2 ions. The reason for the discrepancy is evident in Figures 4.1B, and 4.1C: the median of the arcsine transformed Kodak acids and Refined Merichem data are closer to each other than the median of the logit transformed data for the same samples.

Comparing the two methods for some of the ore samples showed the resulting conclusions differ. Syncrude D was found to differ from Syncrude G because of ions in group 3, but similar to Albian C, Syncrude C, and Syncrude E after arcsine transformation. Logit transformation on the other hand concluded that Albian C, and Syncrude C differed, whereas G and E were similar to Syncrude D. Syncrude D

Table 4.2 Results from two statistical methods used to analyze naphthenic acids: independent samples t-test after arcsine transformation (*italics*) and pairwise comparison after logit transformation (**bold**).

	Kodak acids	Kodak salts	Refined Merichem	MLSBF
Kodak acids		*1,2,3*	*NONE*	*1,3*
Kodak salts	**1,2,3**		*1,2,3*	*3*
Refined Merichem	**2**	**1,2,3**		*1,2,3*
MLSBF	**2,3**	**3**	**2,3**	

comparisons to Syncrude E resulted in similar conclusions from both methods. Such disparity is caused by the differences as to which ions are more influential in the analysis. Arcsine transformation allows highly abundant ions to greatly influence the results, whereas logit transformation makes them less influential, and the presence or absence of an ion becomes more important, as demonstrated by Figure 4.1.

4.3.2 Comparing 15 ore samples

All of the three-dimensional plots and matrices showing proportion of each ion for the ore samples are included in the Appendix. As described in Chapter 3, the data were divided into three groups, based on previous experience. Group 1 is made up of compounds with 5 to 13 carbons, group 2 contain compounds with 14 to 21 carbons, and group 3 contained compounds with 22 carbons or more – the C22+ cluster as determined by Holowenko et al. (2002). Each group for each sample was compared to the same group from the other 14 ore samples using linear mixed models, pairwise comparisons.

The ore samples were from four oil sands companies with mining leases in the Athabasca oil sands region. As shown in Table 4.1, they included Syncrude, True North, Suncor and Albian. The Syncrude samples came from two sites, Aurora (A to E) and the North mine of the Mildred Lake site (Lease 17/22, labeled F and G). Pairwise

Table 4.3 Results from pairwise comparison after logit transformation of the 15 oil sands ore samples: Alb = Albian, Sun = Suncor, Syn = Syncrude, TN = True North. The numbers represent the group considered statistically different ($P<0.05$). Boxes which are blank are not different; groups where the differences were found are labeled. Results are reflected along the diagonal.

	Alb A	Alb B	Alb C	Sun A	Sun B	Syn A	Syn B	Syn C	Syn D	Syn E	Syn F	Syn G	TN A	TN B	TN C
Alb A		3		3	3	3	3	1,3	3	3	3	3	3	3	3
Alb B	3		3	3	3	3	3	3		3	3	3	3	3	3
Alb C		3		3	3	3	3	1,3	1,3	3	3	3	3	3	3
Sun A	3	3	3			3	3	3	3	3	3	3	3		3
Sun B	3	3	3					3	3	3	3	3	3		3
Syn A	3	3	3	3					3			3	3	3	
Syn B	3	3	3	3					3			3	3	3	
Syn C	1,3	3	1,3	3	3				3				3	3	
Syn D	3		1,3	3	3	3	3	3			3		3	3	3
Syn E	3	3	3	3	3								3	3	
Syn F	3	3	3	3	3				3				3	3	
Syn G	3	3	3	3	3	3	3						3	3	
TN A	3	3	3	3	3	3	3	3	3	3	3	3			3
TN B	3	3	3			3	3	3	3	3	3	3			3
TN C	3	3	3	3	3				3				3	3	

comparisons resulted in Table 4.3. Here, the group number where differences were found are shown. Comparisons where no differences were found are blank (i.e. Syncrude A vs. Syncrude B), differences are labeled (i.e. Albian A vs. Albian B differ in group 3; Albian A vs. Syncrude C differ in groups 1 and 3).

After pairwise comparisons of logit transformed data, nearly all of the differences among the 15 samples were due to compounds in group 3 (Table 4.3) as defined in Chapter 3. The only exceptions were in comparisons between Albian A vs. Syncrude C, Albian C vs. Syncrude C, and Albian C vs. Syncrude D. In these cases, both groups 1 and 3 were considered to be statistically different. Comparisons are reflected along the diagonal (black boxes).

The data in Table 4.3 were used to construct Figure 4.2. The reflected data allowed for easier comparison between samples. From Table 4.3, samples whose columns are the same are considered similar and relate to other samples in the same way. Among the 15 ore samples, two such pairs were found; these were Syncrude A and B, as well as Syncrude F and True North C. These pairs are shown in Figure 4.2 in boxes.

Comparisons which resulted in no difference (blank in Table 4.3) are connected by a line in Figure 4.2. Although Syncrude E is considered statistically similar to all other Syncrude samples, not all Syncrude samples were similar to each other. For example, Syncrude C is statistically different from Syncrude D (Figure 4.2). Albian A and C are considered similar to each other, but are not similar to any of the other ore samples (Figure 4.2).

Figure 4.3 shows the results of the hierarchical cluster analysis of the logit transformed data for the 15 ore samples. Cluster coefficients in the agglomeration table were used to determine possible cluster solutions. As shown in Figure 4.3A, a six cluster solution is possible. The clusters would have the following composition: (1) Suncor A, Suncor B, True North B; (2) Syncrude A, Syncrude B; (3) True North A; (4) Syncrude C, Syncrude E, True North C, Syncrude F, Syncrude G, Syncrude D; (5) Albian B; (6) Albian A, Albian C. These groupings can also be detected in Figure 4.2. Other possible solutions include a three cluster solution (Figure 4.3B), where clusters 1, 2, and 3 from the six cluster solution are combined to form one cluster; 4 and 5 forms the second cluster; and 6 the third cluster. The two cluster solution (Figure 4.3C) results from combining clusters 1 through 5 from the six cluster solution to form one cluster, while cluster 6 forms the second cluster. The two cluster solution has the biggest difference between the clusters (from the agglomeration table), followed by the three, and finally the six cluster solution. Comparison of these results to Figure 4.2 shows agreement between the two methods. In both cases, Albian A and C are considered most different from all other samples, and the other groupings can be detected in Figure 4.2. On the other hand, whereas pairwise comparison found Syncrude D to be equally similar to Syncrude G as it is to Albian B, cluster analysis shows that while Syncrude D is in the same cluster as Syncrude G (Figure 4.3A), Albian B is on its own. Syncrude D, G and Albian B are in the same cluster if a three cluster solution is considered (Figure 4.3B).

Cluster analysis and pairwise comparisons differ in how differences between samples are measured. In pairwise comparisons, the mean of the logit transformed data for each of the three groups are compared using the least significant difference method. Cluster analysis does not have as strong a statistical basis as this. It is described as

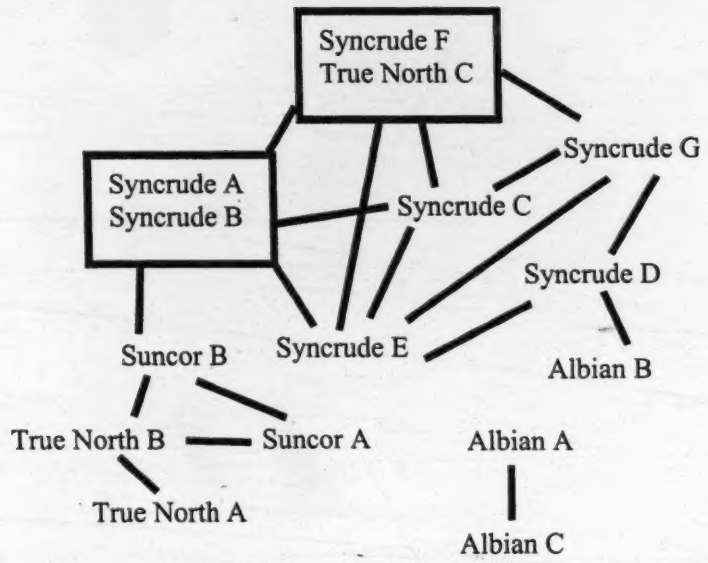

Figure 4.2 Summary of Athabasca oil sands ore sample statistical analysis results (from Table 4.3). Samples connected by a line are considered statistically similar (P>0.05); those in a box are statistically similar and relate to the other samples in the same way.

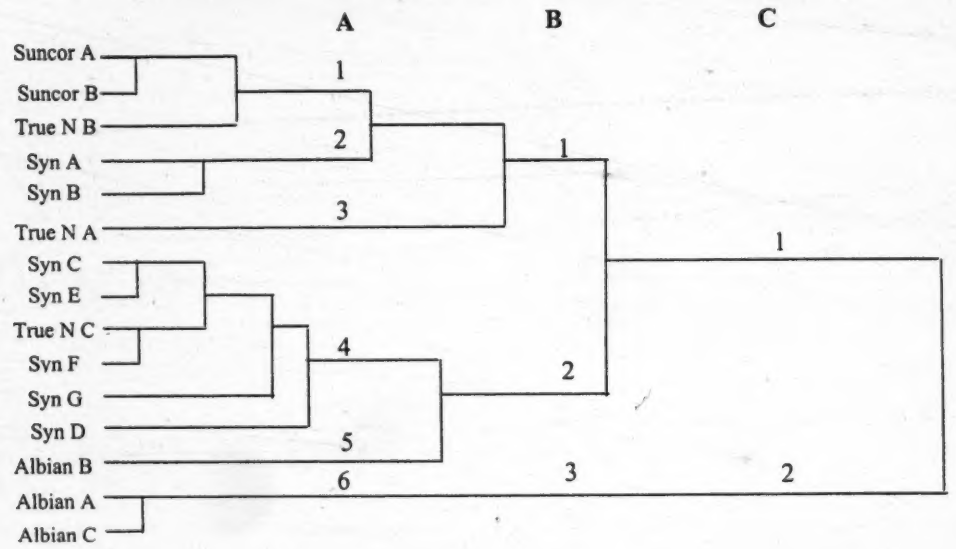

Figure 4.3 Hierarchical cluster analysis (squared Euclidean distance, within group average linkage) of the 15 ore samples. Possible solutions of 6 (A), 3 (B), and 2 (C) clusters are shown.

grouping samples according to rules of thumb, as determined by algorithms (Hair et al. 1984). In my analysis, distance between clusters was calculated using squared Euclidean distance. This means that each of the squared difference between the same ions (same C number, Z family combination) from two samples was summed (Hair et al. 1984). Groups are then determined by measuring the average distance between clusters, where those with smallest distances are combined first. Cluster analysis is therefore more sensitive to differences in the abundance of specific ions because differences are taken before the data are simplified into one value; the mean in the case of pairwise comparisons, the squared Euclidean distance in the case of cluster analysis.

Figure 4.4 gives three examples of three-dimensional graphs compared using these procedures. In it, we see that both True North C and Syncrude F have high ion concentrations in the C22+ cluster, though the majority of their ions lay between 14 and 21 carbons. True North C and Syncrude F are considered similar (Figures 4.2 and 4.4), using pairwise comparisons, and relate to all other ores in the same way. Albian A on the other hand, is considered different from both True North C and Syncrude F (Figures 4.2 and 4.4). It has a large abundance of ions in group 2, but there were very few ions in group 3.

4.3.3 Variables which may affect the observed ion distribution

Since the differences were not delineated by company, other factors may explain the observations summarized in Figures 4.2 and 4.3. Table 4.1 shows the naphthenic acids concentrations of only 12 of the 15 ores, as data for Albian samples were not available. The ores contained different naphthenic acids concentrations, Syncrude E (Aurora transition ore) had the highest concentrations at 500 mg naphthenic acids/kg of oil sand, and Syncrude B had the lowest concentration at 50 mg naphthenic acids/kg oil sand. There was a large variation in the amounts of naphthenic acids from the Syncrude samples. This is in contrast with True North and Suncor samples whose naphthenic acids content were similar across samples for each company. From Figures 4.2 and 4.3 , although the distribution of ions in Suncor A and B were considered to be similar, the same was not true for the True North samples. In Figure 4.2, True North A and B were

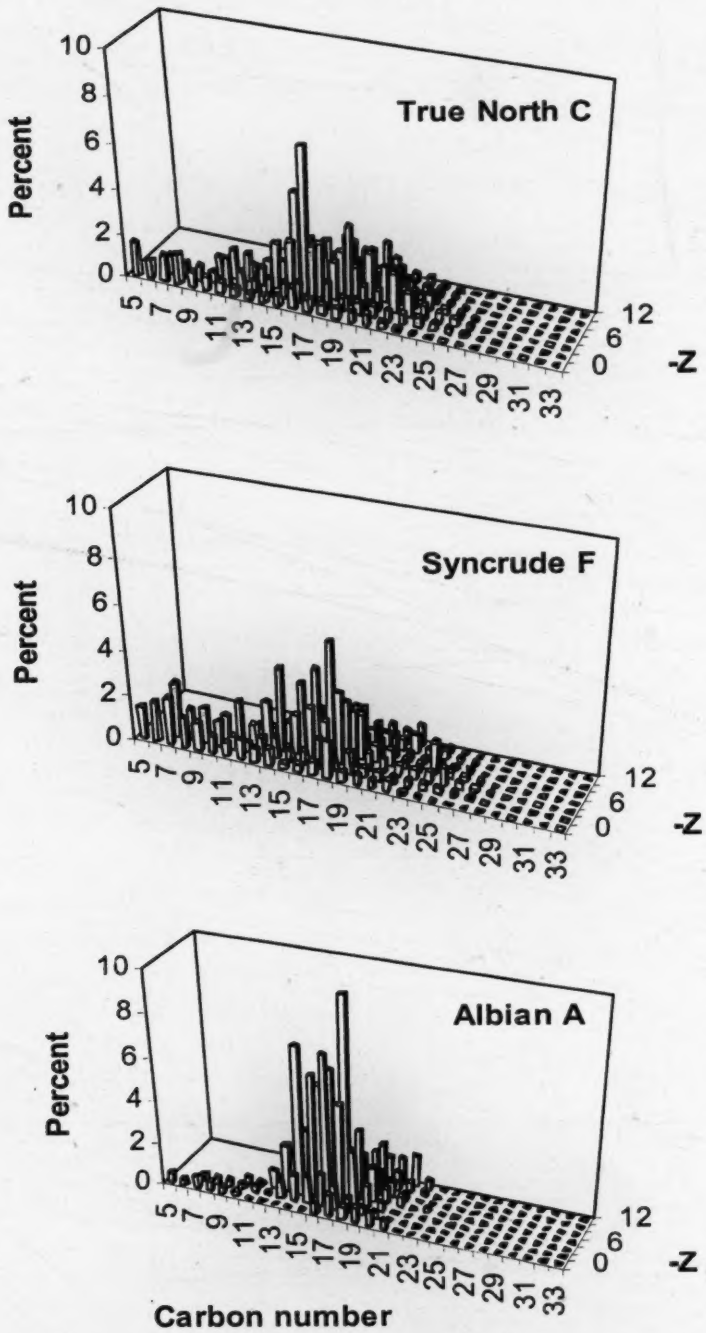

Figure 4.4 Sample three-dimensional graphs analyzed to produce Table 4.3, which includes Syncrude F, True North C, and Albian A.

statistically different from True North C. In addition, Syncrude F was considered statistically similar to True North C. A six cluster solution (Figure 4.3) would consider all True North samples to be different from each other, whereas Syncrude F and True North C are considered to belong to the same cluster. This is despite the fact that Syncrude F and True North C naphthenic acids concentration (Table 4.1) and lease sites were different. Syncrude F has about 320 mg naphthenic acids/kg oil sand, whereas True North C contains about 130 mg naphthenic acids/kg oil sand.

The difference between Syncrude A and B is in their extraction efficiency. Syncrude B gave higher bitumen extraction efficiency than Syncrude A. Since these samples are considered statistically similar (Figure 4.2), and relate to the other samples in the same way, processing and naphthenic acids distribution were not related in the present analysis. Thus, the naphthenic acids distribution (parent ion from GC-MS) was not related to naphthenic acids content in the oil sands (mg/kg), lease site, nor did it appear to be related to the quality of processing, when Syncrude A and B were considered. Processing quality was related to the naphthenic acids concentration. Syncrude B, which underwent better processing than Syncrude A, had a higher naphthenic acids concentration at 149 mg naphthenic acids/kg oil sand compared to Syncrude A, which had 50 mg naphthenic acids/kg oil sand (Table 4.1)

4.3.4 Considerations on comparing the methods

It has been shown that naphthenic acids concentrations in mg/kg oil sand is not a determining factor in the naphthenic acids profile observed by GC-MS, using the pairwise multiple comparison described. Comparison of Syncrude A and B, which are similar areas in the mine, but from different facies or depositional environment, suggests that processing and naphthenic acids distribution are not strongly related. However, the type of statistical analysis employed does affect the groupings derived from the same data (Figures 4.2 and 4.3). Logit transformation stabilizes the variance better than arcsine transformation. The result is that ions with high signals are not weighted as much after logit transformation, compared to when arcsine transform is used. The question then, is whether or not these unusually abundant ions are significant. For example, Syncrude D is

considered similar to Syncrude G, E, and Albian B (Figure 4.2). However, Syncrude D contains two highly abundant ions (16% and 10%) with carbon numbers 13 and 14, and -Z family -4 (m/z = 267, 281 Figure A4, D). These two ions are not as abundant in the other three ore samples. They were visually striking, and dominated the three-dimensional graph of Syncrude D (Figure A4, D).

On inspection of the three-dimensional graphs of these four samples therefore, Syncrude D may be considered different. The question turns into how important such ions are. Albanian petroleum for example, exhibits a high signal corresponding to β-carotane (Sinninghe Damsté and Koopmans 1997). Though this compound is not a carboxylic acid, it is an example of how a dominant ion may identify a sample. Not logit-transforming the data, on the other hand, would result in differences determined by these highly abundant ions, rather than the overall composition of the sample.

The statistical analysis performed after deciding on the appropriate transformation also affects the results. For example, a six cluster solution would consider all True North samples to be different from each other (Figure 4.3), whereas pairwise comparison considers True North B and A to be similar (Figure 4.2). These two samples are placed in the same cluster, if a two or three cluster solution is considered (Figure 4.3).

Because the differences in ion distribution were not traced to differences in lease site, or mg naphthenic acids/kg oil sand, other factors may help explain the observed differences in naphthenic acids distribution. This may include things such as sampling depth, exposure to oxygen, deposit genesis and history. The choice on what data transformation to employ partly depends on the knowledge of other variables. The groupings and connections which come after statistical analysis must be meaningful in light of these other variables. They can therefore help decide as to which data transformation to employ.

Despite some differences in the grouping of the 15 naphthenic acid samples from oil sands ores (Figures 4.2 and 4.3), there is generally good agreement between the pairwise and cluster analysis methods. Although some refinements of the methods may be required to obtain the "true" groupings, the results presented in this chapter represent the first time that the naphthenic acids composition of so many ore samples have been determined. It is also the first time that statistical methods have been applied to compare

and group samples analyzed by MS. These results provide an excellent first step toward understanding the differences and similarity among the naphthenic acids compositions of various oil sands ores.

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5. EXTRACTION OF NAPHTHENIC ACIDS FROM AQUEOUS SOLUTION

5.1 Introduction

Naphthenic acids are a group of chemically and structurally related alkanoic acids with the general formula $C_nH_{2n+Z}O_2$, where n is the carbon number and Z is related to the number of rings. They may be cyclic or acyclic. The cyclic components are also substituted with an alkyl. The acyclic components, unlike fatty acids, are highly branched (Rudzinski et al. 2002). These compounds have surfactant properties, with acid dissociation constants of 10^{-5} to 10^{-6} (Brient et al. 1995). They are natural components of crude oils (Seifert and Teeter 1969, Seifert et al. 1969, Fan 1991, Tomczyk et al. 2001), at concentrations that range between 0.0015% in Egyptian crude, and 2% in Athabasca oil sands (CONRAD 1998, Brient et al. 1995).

This group of compounds has commercial applications as wood preservatives, and surfactants. Isolation for commercial purposes is achieved by extracting the petroleum fraction which distils at 200 °C to 370 °C with 2% to 10% NaOH (Brient et al. 1995). The naphthenic acids are then recovered from the alkaline solution and refined by a proprietary process.

Naphthenic acids are also extracted from bitumen as a consequence of oil sands ore operations. Bitumen is extracted from ores by exposing the oil sands to steam, hot water, and NaOH. This treatment solubilizes the naphthenic acids, making them part of the wastewater (Syncrude 2000). Process-affected waters are stored in closed ponds, such as the Mildred Lake Settling Basin (MLSBF), which was created in 1978 (MacKinnon 1989). The basin has a volume of about 200 x 10^6 m^3 (Leung 2001). Naphthenic acid concentrations in ponds range between 20 mg/L (when storage was undisturbed) to 120 mg/L in ponds that receive continuous input of tailings waste (Holowenko et al. 2002).

To analyze naphthenic acids using the method developed by St. John et al. (1998), they must be in organic solvent (CH_2Cl_2), in their acid form. Naphthenic acids from oil

sands wastewaters have been extracted using two methods. The first involves adsorption and desorption to solids, which was facilitated by changing the pH, as well as settling and centrifugation (Holowenko et al. 2001). Another method involves acidifying the tailings water to a pH of ~2.0, and using dichloromethane as an extraction solvent (Rogers et al. 2002).

Naphthenic acids are composed of hundreds of carboxylic acids, and the partition coefficients of these would differ depending on molecular size. Dichloromethane extractions of sea water containing fatty acids with different numbers of carbon atoms yielded different recoveries (Osterroht 1993). For example, Osterroht (1993) found 38% recovery for C5, 85% recovery for C18, and 93% recovery for C16 and C14 fatty acids. Thus, different solvent combinations were evaluated in an attempt to optimize naphthenic acids extraction efficiency. One of the objectives of this project is to demonstrate changes in the naphthenic acids compositions as a result of microbial activity. These changes were to be determined by comparing GC-MS analyses of naphthenic acids extracted from laboratory cultures. I, therefore, had to ensure that the method of extraction was reliable.

In addition, an internal standard was sought as a qualitative indication of any decrease in naphthenic acids concentrations during biodegradation. This was because both Kodak salts and Merichem acids, which would be used in subsequent biodegradation experiments (Chapter 6), do not contain a high proportion of compounds with 22 to 33 carbons. It was unlikely that their three-dimensional plots would change. The increase in the proportion of compounds in the C22+ cluster, as described by Holowenko et al. (2002), was not expected to occur.

5.2 Materials and Methods

5.2.1 Materials

Dichloromethane (HPLC grade, Fisher, Fair Lawn, NJ); glass distilled ethylacetate (Caledon, Georgetown, ON), pentane (Caledon), acetone (reagent grade, Fisher), and glacial acetic acid (Mallinckrodt, Phillipsburg, NJ), were used for liquid-liquid extractions. The activity of ^{14}C-palmitic acid in hexane (Amersham Biosciences,

UK) was determined to be 2.2 x 10^7 dpm/mL (disintegrations per minute/mL). Non-radioactive palmitic acid (Aldrich Chemical Co, Milwaukee, WI) was used to test several extraction solvents. Saturated salt solution consisted of NaCl in double distilled water.

Extraction solvent mixtures for the quantitative study included (v/v): ethylacetate with 1% acetic acid; ethylacetate with 2% acetic acid; dichloromethane; and dichloromethane with 1% acetic acid.

5.2.2 General methods for liquid-liquid extractions

Prepared standards were extracted three times using a 1:2 ratio of organic solvent to aqueous sample; typically 10 mL of solvent to 20 mL of sample. To compare the extraction efficiencies of different organic solvents, a solution of 100 mg Kodak salts/L was prepared in 0.025 M NaOH, which gave a pH of approx. 12. This was adjusted to approx. pH 2 with 0.5 mL of 2 M sulfuric acid when the samples were to be analyzed by HPLC or GC-MS, or with 0.25 mL of 4 M sulfuric acid when the samples were analyzed for radioactivity. One milliliter of saturated NaCl solution was added to the 20-mL samples that were to be extracted with ethylacetate solutions. Because of the relatively high solubility of water in ethylacetate, pooled ethylacetate extracts were washed with 10 mL of saturated NaCl solution to reduce the amount of water remaining in the organic solvent. The organic layers were pooled and prepared for derivatization.

5.2.3 HPLC analyses of organic extracts

The individual and the pooled organic extracts containing naphthenic acids were dried over anhydrous sodium sulfate for about 1 h. To determine the amount of naphthenic acids extracted into the organic solvent, a 50-µL sample of the organic solvent was removed and dried under a flow of nitrogen. This sample was re-dissolved in 50 µL ethanol and analyzed as in Chapter 2, using half the reagent volumes. The percent recovery was determined by comparing the measured naphthenic acids concentration to the expected concentration after taking into account for 9% sodium in the salt sample. Kodak acid was used for the calibration curve.

5.2.4 *Extraction of radioactive palmitic acid*

The efficiencies of liquid-liquid extractions with various solvents were determined using ^{14}C-palmitic acid. For each extraction, 20 mL of a solution of 100 mg Kodak salts/mL in NaOH (pH 12) was placed in a separatory funnel and then 100 µL of ^{14}C-palmitic acid in hexane (2.2×10^7 dpm/mL) was added to the aqueous layer. This was mixed and allowed to stand for 15 min to evaporate hexane. A 100-µL sample was taken from the aqueous layer to determine the actual amount of radioactivity added to the separatory funnel. Then the remainder of the solution in the separatory funnel was acidified to pH 1.5 with 0.2 mL of 4 M sulfuric acid. Three organic extraction solvents were tested individually. These were dichloromethane, 1% acetic acid in dichloromethane, and 2% acetic acid in ethylacetate. Each 20-mL portion of the Kodak salts solution was extracted three times with 10-mL portions of fresh solvent. After each extraction, a 100-µL sample was removed from the aqueous layer to determine the amount of residual radioactivity.

The three portions of organic solvent were pooled and a 100-µL sample was removed to determine the amount of extracted radioactivity. The dichloromethane in the liquid scintillation counting vial was evaporated to dryness to avoid chemical quenching during radioactivity counting. Each vial received 10 mL of ASC fluor (Amersham) and the amount of activity (dpm) was determined using a Beckman LS 3801 liquid scintillation counter. Percent recovery of ^{14}C-palmitic acid was determined by comparing the dpm prior to extraction, after each extract, and in the organic phase, after accounting for differences in volumes.

5.2.5 *Evaluations of internal standards for GC-MS*

Two possible internal standards were tested to provide a semi quantitative indication of the decrease in naphthenic acids concentrations. The compounds tested were *trans*-cinnamic acid (Aldrich), and 5β-cholanic acid (Sigma Chemicals Co., St. Louis, MO). Separate solutions (8 mM) of each acid were prepared in dichloromethane and a solution of Kodak acids (30 mg/mL) was also prepared in dichloromethane. The

reaction for GC-MS analysis consisted of a 50 μL portion of the internal standard, and 50 μL of the sample.

To test the ability to recover the internal standard from the liquid culture medium, 0.33 mL of 0.53 mM *trans*-cinnamic acid (175 nmol) dissolved in 0.025 M NaOH was added to 20 mL of Kodak salt at 100 mg/L (2 mg Kodak salt) dissolved in modified Bushnell-Haas medium (Wyndham and Costerton 1981). The pH was adjusted to approximately 1.5 with sulfuric acid, and 1 mL of saturated NaCl solution was added. Then, this mixture was extracted twice with 10 mL of ethylacetate containing 2% (v/v) acetic acid, as described previously. The solvent was evaporated to dryness and the residue was dissolved in 100 μL of dichloromethane and the acids were derivatized, and analyzed by GC-MS as outlined by Holowenko et al. (2002).

5.2.6 GC-MS analysis of organic extracts

Extracts to be derivatized for gas chromatography – mass spectrometry (GC-MS) analysis were dried over anhydrous sodium sulfate for about 1 h, and then filtered to remove the drying agent. The solvent was removed under vacuum using a rotavapor R11O (Brinkmann, Rexdale, ON). The residues were transferred to 1.5-mL dram vials using dichloromethane, and taken to dryness under a flow of nitrogen. Then the residue was dissolved in 100 μL of dichloromethane, derivatized to their *t*-butyldimethylsilyl esters and analyzed using GC-MS as outlined by Holowenko et al. (2002).

5.3 Results

5.3.1 Survey of solvents for extraction of naphthenic acids

A quick procedure using a non-radioactive palmitic acid suspension was used to determine which of nine solvent combinations were suitable for more extensive extraction trials with naphthenic acids. Solvents that were able to dissolve palmitic acid, and had two phases after addition to the palmitic acid suspension were further tested. Those solvents that gave more polar mixtures gave positive results. These included:

dichloromethane; ethylacetate; 1% (v/v) acetic acid in dichloromethane; and 1% (v/v) acetic acid in ethylacetate. The ethylacetate extractant was changed to contain 2% (v/v) acetic acid to improve naphthenic acids recovery.

5.3.2 Recovery of naphthenic acids from aqueous solutions by liquid-liquid extraction

The recovery of ^{14}C-palmitic acid, a surrogate naphthenic acid, mixed with a solution of 100 mg Kodak salts/L is summarized in Table 5.1. About 2.7 x 10^6 dpm of ^{14}C-palmitic acid was added to each of three naphthenic acids solutions, and each was extracted three times with one of the three solvents described previously.

Extraction with 2% acetic acid in ethylacetate gave higher recoveries of ^{14}C-palmitic acid than extractions with dichloromethane (Table 5.1). Table 5.1 indicates that extraction with 2% acetic acid in ethylacetate gives a higher final recovery, with only 1.5% of the radioactivity remaining in the aqueous phase, compared to 5% in those extracted with dichloromethane and 1% v/v acetic acid in dichloromethane. Only 3% of the original radioactivity was left in the aqueous phase after the first extraction with 2% acetic acid in ethylacetate, compared to 37% left after one extraction with dichloromethane and 18% left after one extraction with 1% acetic acid in dichloromethane (data not shown). Therefore, 2% acetic acid in ethylacetate not only gives the highest recovery, but was also more efficient than extractions with the other two methods that used dichloromethane.

The data in Table 5.1 are the results of a single determination for each sample containing ^{14}C-palmitic acid. Further experiments were done to compare the reproducibility of two extraction methods; one using dichloromethane, and the other using 2% acetic acid in ethylacetate. Three 20-mL portions of 100 mg Kodak salts/L were extracted and the amounts of the extracted naphthenic acids were determined by HPLC. The results are summarized in Table 5.2. They show that the range of recoveries using dichloromethane or 2% acetic acid in ethylacetate were essentially the same. However, the extraction with 2% acetic acid in ethylacetate was more reproducible with a relative standard deviation of 3% compared to 7.6% with dichloromethane.

Table 5.1 Comparison of the recoveries of radioactivity during the extraction of ^{14}C-palmitic acid in 20 mL of 100 mg Kodak salts/L (pH approx. 1.5) using three different solvents.

	Extraction solvent		
	Dichloro-methane	1% Acetic acid in dichloromethane	2% Acetic acid in ethylacetate
Initial aqueous count (dpm)	2,878,600	2,678,800	2,726,400
Aqueous count after three extractions (dpm)	157,800	128,400	41,400
Count left in aqueous phase (%)	5	5	2
Counts in pooled organic extracts (dpm)	2,586,300	2,608,200	2,784,300
Counts recovered in organic phase (%)	90	97	102

Table 5.2 Recovery and reproducibility of extraction of 20-mL portions of 100 mg Kodak salts/L using dichloromethane and 2% acetic acid in ethylacetate as solvents.

Solvent	Average recovery (%)[a]	Range of recovery (%)	Relative std dev. (%)
Dichloromethane	94	90-103	8
2% Acetic acid in ethylacetate	98	95-101	3

[a] Three replicate samples extracted.

Anhydrous sodium sulfate, the drying agent, does not absorb acetic acid; the drying agent remains loose and powdery when added to ethylacetate containing 2% (v/v) acetic acid. The extracted and salt-washed samples were derivatized and analyzed via GC-MS to determine if different extraction procedures would give different ion distributions (Table 5.3). Statistical comparisons showed there were no significant differences in the distributions of ions in the naphthenic acids obtained from the extraction with dichloromethane or 2% (v/v) acetic acid in ethylacetate. That is, all the P-values were greater than 0.05.

Table 5.3 Results from statistical analyses of the naphthenic acids in Kodak salts and MLSBF naphthenic acids extracted from aqueous solution with ethylacetate containing 2% acetic acid (v/v) or with dichloromethane.

Naphthenic acids	Group	P[b]	Sums[a]	
			2% Acetic acid in ethylacetate	Dichloromethane
Kodak salts	C5 to C13	0.911	21.2	22.0
	C14 to C21	0.960	78.1	77.2
	C22 to C33	0.860	0.66	0.75
MLSBF	C5 to C13	0.693	17.4	14.6
	C14 to C21	0.915	82.5	85.4
	C22 to C33	0.579	0.097	0.047

[a] Total ion abundance in each specified group.
[b] Two samples are considered significantly different if $P<0.05$.

5.3.3 Selecting an internal standard for GC-MS analyses

Two compounds were tested as suitable internal standards. These were *trans*-cinnamic acid, an aromatic (C=9, Z=-10) and 5β-cholanic acid, a steroid (C=24, Z=-8). Cinnamic acid falls outside the accepted range for naphthenic acids (cell with diagonal line in Tables 1.5, and 1.6), whereas ions with the C and Z numbers of 5β-cholanic acid are very rarely seen in the naphthenic acids that I have examined (e.g. Kodak acids and salts, Merichem acids, and the MLSBF naphthenic acids).

As shown in Figure 5.1A, 5β-cholanic acid elutes at 36.5 min, far from the Kodak acids hump. The three-dimensional plot of the ion abundance from this GC-MS analysis shows 5β-cholanic acid at C=24, Z=-8, away from the other ions from Kodak acid. From these GC-MS results, both *trans*-cinnamic acid and 5β-cholanic acid were good candidates for use as internal standards. The internal standard would be added just prior to the extraction of residual naphthenic acids from the culture. Unfortunately, 5β-cholanic acid is not soluble in a dilute, aqueous NaOH solution (pH 12), and therefore is difficult to use with aqueous culture medium.

Thus, *trans*-cinnamic acid, which is soluble in dilute NaOH solutions, was further investigated as a possible internal standard. Mixtures of *trans*-cinnamic acid and Kodak

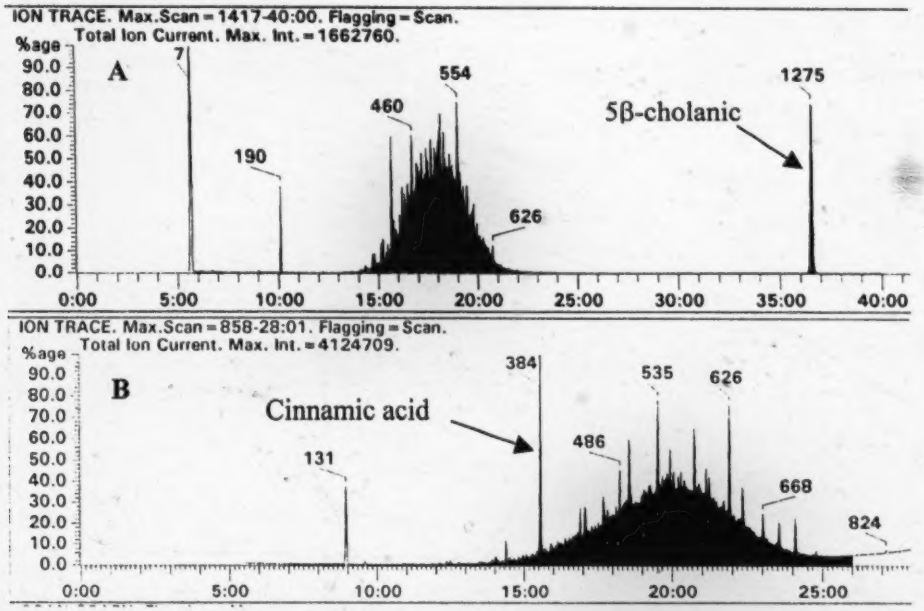

Figure 5.1 Total ion current of a mixture of 5β-cholanic acid (A), and *trans*-cinnamic acid (B) with Kodak salts after extraction from aqueous solution.

acids were derivatized and analyzed by GC-MS. The ion (m/z=205) corresponding to the aromatic standard, *trans*-cinnamic acid, occurs at C=9, Z=-10. Cinnamic acid was successfully co-extracted with Kodak salts from an aqueous solution. After derivatization, GC-MS analysis gave the chromatogram shown in Figure. 5.1B with *trans*-cinnamic acid eluting at 15.5 min in the initial part of the Kodak salts hump.

5.4 Discussion

Schmid (1973) found a chloroform-methanol mixture to be efficient at extracting lipids from biological materials. The solubility parameter resulting from this mixture is not unique because the same efficiency was also found with toluene-ethanol (Schmid et al. 1973), and ethylacetate-acetone (Slayback 1977). I believe that the presence of acetic acid may facilitate formation of acid dimers through hydrogen bonding, which in turn increases the solubility of naphthenic acids in the organic solvents. It is known that naphthenic acids dimerize in dichloromethane because characteristic absorbances are observed in FT-IR scans (Jivraj 1995). In addition, Schmid (1973) reported that the

solubility parameter for acetic acid monomer is 13.01 and that of dimer is 9.19. The increase in solubility parameter observed by Schmid (1973) is caused by the decrease in polarity of dimers. In our system, there should be 3.5 mmol of acetic acid in 10 mL solvent (i.e. the first extract), and 2 mg of naphthenic acid (about 0.01 mmol) in 20 mL solution with 100 mg naphthenic acids/L. This large excess of acetic acid should promote dimer formation between the naphthenic acid and the acetic acid molecules. Dimer formation results from hydrogen bonding between oxygens and hydrogens in the carboxylic groups of two carboxylic acids. These will likely be preferentially dissolved in the organic phase, thereby improving the extraction efficiency, and reproducibility (Tables 5.1 and 5.2).

Acetic acid was not expected to interfere with either the GC-MS or HPLC derivatization procedures because the reagents are in excess and any trace amounts of derivatized acetic acid will elute from the chromatography columns well before the naphthenic acids. Acetic acid has a high boiling point at 118 °C, but can be removed at room temperature under a stream of nitrogen, because only a small volume would be present in the extract. A 30 mL volume of solvent, containing 2% acetic acid would only contain 600 μL of acetic acid in the pooled extract. The results presented in this section demonstrated that the extraction method using 2% acetic acid in ethylacetate is well suited for the recovery of naphthenic acids from aqueous solutions for subsequent GC-MS analyses. However, this method is not suitable for extraction of naphthenic acids for FT-IR analyses. Any residual acetic acid in the extract adversely affects the results from this procedure.

It has been shown that *trans*-cinnamic acid should be a suitable internal standard in the extraction of naphthenic acids from biodegradation experiments. Water solubility of the compound allows it to be easily added to the mineral medium. In addition, the computer program used for the analysis of naphthenic acids (described in Holowenko and Fedorak 2001) ignores m/z=205 because this ion does not fit the working definition of a naphthenic acid (Holowenko et al. 2002). This would provide a qualitative indication of the extent of biodegradation. No attempt was made to use this internal standard for quantitative determination of the amounts of each ion found in the naphthenic acids mixtures.

Internal standards were not used as a semi quantitative indication of changes in naphthenic acids concentrations in MS analysis in subsequent experiments. This was because subsequent experiment showed that the MS program the *trans*-cinnamic acid peak was not consistently included in the average when the 1% minimum occurrence variable was used.

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6. AEROBIC BIODEGRADATION OF TWO COMMERCIAL NAPHTHENIC ACIDS[♣]

6.1 Introduction

Naphthenic acids are found in many petroleums (Lochte and Littmann 1955, Fan 1991, Brient et al. 1995, Slavcheva et al. 1999, Jones et al. 2001) and are a concern because of their potential to cause corrosion during oil refining processes (Slavcheva et al. 1999) and their toxic character in aquatic systems (CONRAD 1998). Naphthenic acids are released from petroleum into alkaline aqueous solutions and are present in wastewaters at petroleum refineries (Dzidic et al. 1988, Wong et al. 1996) and in the extraction waters at the oil sands extraction plants in northeastern Alberta, Canada, where the separation of the bitumen from oil sand is based on an aqueous digestion at elevated pH (MacKinnon and Boerger 1986, Schramm et al. 2000). Their concentration range is approximately 40 to 120 mg/L in the oil sands tailings waters (Schramm et al. 2000, Holowenko et al. 2000, 2001).

Naphthenic acids and metal naphthenates have many commercial uses, including textile and wood preservation, paint driers, emulsifiers, surfactants, and adhesion promoters in tire manufacture (Brient et al. 1995). Naphthenates are less likely to cause long-term damage than other forms of wood preservatives, such as pentachlorophenol, creosote, and chromated copper-arsenic (Brient et al. 1995). Naphthenic acids are surfactants and have been shown to be acutely toxic to a variety of organisms (Dokholyan and Magomedov 1984, MacKinnon and Boerger 1986, CONRAD 1998, Dorn 1992, Rogers et al. 2002). This toxicity is an issue facing the oil sands processing companies, both in terms of water management and for reclamation options of disturbed areas in northeastern Alberta. Previous studies have shown that the toxicity of oil sands tailings waters is reduced by natural aging (MacKinnon and Boerger 1986, Holowenko et al. 2002) and by aerobic microbial activity in laboratory cultures (Herman et al. 1994, Lai et al. 1996, Moore et al. 2002).

[♣] A version of this chapter has been accepted for publication. Clemente, J.S., M.D. MacKinnon, and P.M. Fedorak. November 2003. Environ. Sci. Technol.

Naphthenic acids comprise a complex mixture of alkyl-substituted acyclic and cycloaliphatic carboxylic acids, with the general chemical formula $C_nH_{2n+Z}O_2$, where n indicates the carbon number and Z is zero or a negative, even integer that specifies the hydrogen deficiency resulting from ring formation. Typical structures are given in Chapter 1, Figure 1.1.

Various mass spectrometry methods (Dzidic et al. 1988, Fan 1991, Wong et al. 1996, Morales-Izquierdo 1999, Hsu et al. 2000) and gas chromatography-electron impact mass spectrometry (GC-MS) methods (St. John et al. 1998, Holowenko et al. 2001, 2002) have been used to qualitatively characterize the types of compounds in naphthenic acids from different sources. Recently, GC-MS methods have been used to determine the relative distributions of compounds in naphthenic acids in commercial preparations (St. John et al. 1998, Holowenko et al. 2001) and in various process-affected water samples from two oil sands extraction plants (Holowenko et al. 2001, 2002). To date, there is no analytical method that will separate the individual compounds found in the complex mixture of naphthenic acids.

Holowenko et al. (2002) used three-dimensional graphs (with relative ion intensities plotted as a function of carbon number and Z value) to illustrate the distribution of ions (from GC-MS analyses) with m/z values that are consistent with structures of naphthenic acids. This presentation provides a "fingerprint" of a naphthenic acids preparation. They observed few naphthenic acids with carbon numbers 20 to 22 in the samples that were examined. Thus, Holowenko et al. (2002) defined the C22+ cluster to be the sum of all naphthenic acids with carbon numbers of 22 to 33 in Z families 0 to -12. This parameter proved to be extremely useful for comparing water samples that had a range of toxicities (IC_{20} values measured by the Microtox™ method). A decrease in toxicity of oil sands process-affected waters accompanied an increase in the proportion of naphthenic acids in the C22+ cluster. From their examinations of oil sands process-affected waters, Holowenko et al. (2002) postulated that biodegradation of lower molecular weight naphthenic acids occurred preferentially, thereby increasing the relative proportion of the acids in the C22+ cluster. This hypothesis suggests that a decrease in the relative proportion of lower molecular weight acids reduces the toxicity of the residual naphthenic acids.

This study examined the relative susceptibilities of lower molecular weight naphthenic acids and higher molecular weight naphthenic acids to microbial degradation. Two commercial naphthenic acids preparations were studied, and the impact on the acute toxicity with the change in naphthenic acids composition was determined. Biodegradation was monitored by measuring the decrease in naphthenic acids concentrations by high performance liquid chromatography (HPLC), the production of CO_2 from the naphthenic acids (known as mineralization), and the change in the proportions of compounds with various n and Z numbers (by GC-MS).

6.2 Materials and Methods

6.2.1 Naphthenic acids used

Kodak naphthenic acids sodium salt ("Kodak salts") (lot B14C) was purchased from The Eastman Kodak Company (Rochester, NY). By weight, the salt contained 9% sodium (Chapter 2). Refined naphthenic acids ("Merichem acids") were provided by Merichem Chemicals and Refinery Services LLC (Houston, TX). They had an acid number of 257 mg KOH/g, with a clear golden color (3 minimum on Gardner scale) and they contained 4% to 8% (w/w) of unsaponifiables (generally hydrocarbons and phenols). In preparation for the mineralization experiments, subsamples of the two commercial products were analyzed for carbon content in the Microanalytical Laboratory, Department of Chemistry, University of Alberta. The Kodak salts were 64.8% carbon and the Merichem acids were 73.4% carbon.

6.2.2 Culture methods

Enrichment cultures of naphthenic acids-degraders were established with each of the commercial naphthenic acids preparations. The source of microorganisms was process-affected water from the Mildred Lake Settling Basin (MLSBF), a containment pond for oil sand extraction tailings at the Mildred Lake site of Syncrude Canada Ltd. The enrichment cultures were established in 500-mL Erlenmeyer flasks that contained

200 mL of sterile modified Bushnell-Haas mineral salts medium (Wyndham and Costerton 1981) (pH approximately 7), inoculated with 20 mL of the tailings pond water. The naphthenic acids preparations (about 100 mg/L) served as the sole carbon source. The enrichment cultures were incubated under aerobic conditions in the dark on a shaker at room temperature (about 21 °C). At monthly intervals, over several months, portions of the cultures were transferred (1% v/v) to fresh medium and the corresponding naphthenic acids preparation.

Biodegradation experiments were done to monitor the loss of the commercial naphthenic acids from viable cultures, the mineralization of these acids, the changes in the composition of the naphthenic acids, and the reduction of toxicity of the residual compounds. The cultures were incubated in 125-mL serum bottles. During incubation, the top of each bottle was also sealed with a serum stopper to make a gas-tight incubation system so that none of the microbially produced CO_2 could escape. Headspace gas and the liquid culture medium were sampled using a syringe, without removing the serum stoppers. The amount of oxygen in the headspace gas (at the time of inoculation) was sufficient for the complete mineralization of the naphthenic acids solutions (Chapter 2).

A stock solution of the Kodak salts was prepared at 2180 mg/L in 0.1 M NaOH. Each mineralization culture was prepared by adding 2 mL of the filter-sterilized (using Durapore membrane filter, GV, 0.22 μm, Millipore, Bedford MA) stock solution to 38 mL of modified Bushnell-Haas medium and 0.4 mL of viable inoculum from the culture enriched on the Kodak salts. This gave a final volume of 40 mL with the naphthenic acids, as the naphthenates, at 98 mg/L. Thirty replicate cultures were prepared for each experiment. In addition, one set of sterile controls contained 38 mL of medium, 2 mL of the stock naphthenic acids solution, and 0.4 mL of the heat-killed enrichment culture. Another set of sterile controls was prepared in the same manner with 2 mL of 0.1 M NaOH replacing the stock naphthenic acids solution. Exactly the same protocol was used for the cultures incubated in the presence of the Merichem acids (initial concentration 102 mg/L). An enrichment culture that received Merichem acids as its sole carbon source was used as the inoculum for this set of cultures.

The viable cultures and controls were incubated under aerobic conditions, in the dark, on a shaker at 200 rpm at room temperature. At each sampling time, portions of

three viable cultures were analyzed by HPLC, and for mineralization products (dissolved organic carbon and CO_2 in the headspace). At selected times, triplicate controls were sampled and analyzed using the same methods. At each sampling time, a portion of one of the triplicate viable cultures was stored for GC-MS analysis, and one to three of the replicate cultures were saved for Microtox™ assay. Collected samples were stored at -20°C until they were analyzed.

6.2.3 Plate count used to determine microbial growth

To determine the numbers of viable microorganisms in the naphthenic acids-degrading cultures, a sterile syringe and needle were used to remove a 1-mL sample from a sealed culture bottle. This was serially 10-fold diluted in sterile phosphate buffer (10 mM, pH 7) and 0.1 mL aliquots were plated in R2A agar (Difco, Becton-Dickinson, Sparks, MD) in triplicate. At the same time, samples (0.1 mL) from sterile controls were plated (without serial dilution) to confirm sterility. The inoculated plates were incubated for 2 weeks, in the dark at room temperature prior to counting colony forming units (cfu).

6.2.4 Naphthenic acids quantitation by HPLC

The derivatization protocol and the HPLC method are described in detail in Chapter 2. Briefly, 50 µL of supernatant from the culture were mixed with 100 µL of acidic, 0.02 M 2-nitrophenylhydrazine (ICN Biomedicals Inc. Aurora, OH) solution and 100 µL of 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide hydrochloride (Sigma Chemicals Co., St. Louis, MO) solution, in a sealed 1.5-mL, screw-cap vial. This reaction mixture was heated in a water bath at 60 °C for 20 min. Vials were removed from heat and 50 µL of 69 mM KOH were added. The reaction mixture was heated for another 15 min at 60 °C. The resulting derivatized samples were cooled on ice prior to HPLC analysis.

6.2.5 *Monitoring mineralization*

Dissolved inorganic carbon and CO_2 in the headspace were measured using the method of Bressler et al. (1999). Briefly, 2 mL of liquid and 4 mL of headspace gas (5% of the volume of each phase) were withdrawn from the sealed culture bottles using a syringe. Each sample was injected into a sealed 35-mL serum bottle containing 2 mL of 2 M H_2SO_4. After equilibrating overnight, a 0.5 mL sample of the headspace gas was analyzed by GC for the resulting CO_2 (Bressler et al. 1999). Standards for calibration curves were prepared by adding known amounts of $NaHCO_3$ in Tris buffer (pH 8) into empty serum bottles. These were sealed, and treated in the same manner as the sample from the cultures.

6.2.6 *Analyses of naphthenic acids by GC-MS*

To extract the naphthenic acids, 15 mL of thawed culture sample were centrifuged at 12,000 x g for 15 min, and then 10 mL of supernatant were acidified to pH<2 with 2 M H_2SO_4. This solution was extracted twice with fresh 5 mL portions of ethylacetate containing acetic acid (2% v/v). The organic extracts were combined and washed with 5 mL of saturated NaCl solution, dried with anhydrous Na_2SO_4, and the ethylacetate was removed leaving a residue of naphthenic acids. These were derivatized as outlined by St. John et al. (1998) using *N*-methyl-*N*-(*t*-butyldimethylsilyl)trifluoroacetamide containing 1% *t*-butyldimethylsilylchloride (Sigma, St. Louis, MO).

The GC-low resolution MS method was described previously (Holowenko et al. 2002). The average mass spectrum of the unresolved hump of naphthenic acids was determined (Holowenko et al. 2002). The "minimum occurrence" variable for the averaged data was set at 1%, meaning that an ion had to occur in at least 1% of the total scans averaged to be included in the final average data outputted from the computer.

6.2.7 *Microtox™ assays*

Samples for Microtox™ analyses were stored in 4-mL glass vials at -20°C. After

thawing, the samples were filtered through 0.22 μm filters (Millex-GS, Millipore, Bedford MA), and the filtrates were analyzed by the Basic protocol, according to the Microbics Corp. (Microbics Corporation 1991) manual. A Microbics model M500 was used to read the light output from the reconstituted *Vibrio fischeri*. Luminescence was measured before and at 15 min after adding 500 μL of serially diluted filtrate to 500 μL diluent containing 10 μL of the *V. fischeri*. The results are expressed as IC_{50}, the percent of culture filtrate (v/v) that decreased the photoluminescence by 50%.

To ensure reliability of the Microtox™ method, a toxicity test using phenol (100 mg/L) was done prior to assaying of each set of samples from the biodegradation experiments. As recommended by Microbics Corp. (Microbics Corporation 1992), the observed IC_{50} of phenol fell in the range 13 to 26 mg/L.

6.3 Results

6.3.1 Biodegradation of Kodak salts

After an initial lag time of about 3 days, the concentration of Kodak salts naphthenic acids decreased quickly (Figure 6.1A), and by day 12, >90% were consumed. The concentrations of naphthenic acids in the sterile controls changed little over the 42 days of incubation (Figure 6.1A). The initial bacterial count was 3.1×10^4 cfu/mL, and this increased to 2.3×10^6 cfu/mL on day 3 and remained essentially constant over the remainder of the incubation. Biodegradation of the naphthenic acids was accompanied by the formation of inorganic carbon in the viable cultures. The mineralization results, shown in Figure 6.1A, were corrected for background inorganic carbon measured in the sterile controls (containing naphthenic acids), which remained unchanged over the 42 days. Approximately 60% of the carbon from the Kodak salts was detected as CO_2.

During biodegradation of the naphthenic acids in the Kodak salts, the acute toxicity was reduced (Figure 6.2A). Higher IC_{50} values (expressed as volume percent of diluted culture supernatant), indicate lower supernatant toxicity. At time zero, the viable enriched culture and two control cultures were analyzed by the Microtox™ method (Figure 6.2A). The sterile control that contained heat-killed cells and naphthenic

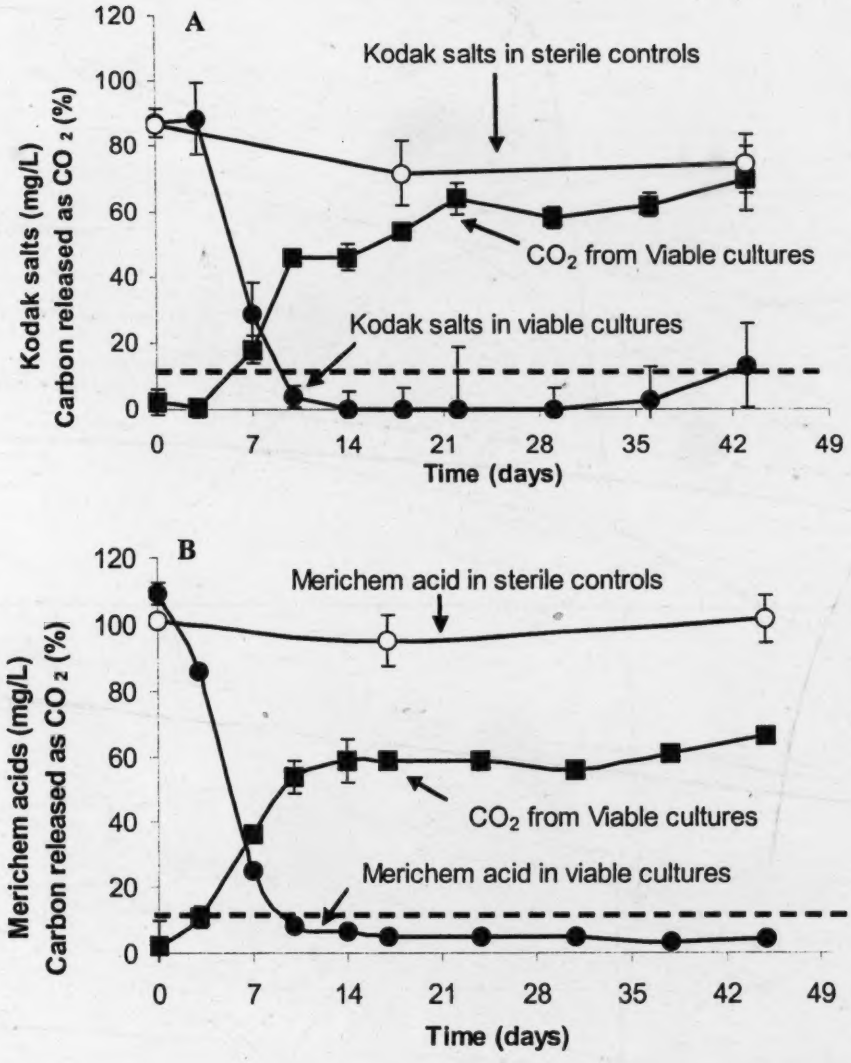

Figure 6.1 The aerobic biodegradation of naphthenic acids in Kodak salts (A), and Merichem acids (B) measured by the decrease in naphthenic acids concentration and the release of CO_2 as the acids were mineralized. Naphthenic acids concentrations of <15 mg/L cannot be accurately determined by the HPLC method used (Chapter 2). The broken line indicates those concentrations <15 mg/L. Error bars are one standard deviation (n=3).

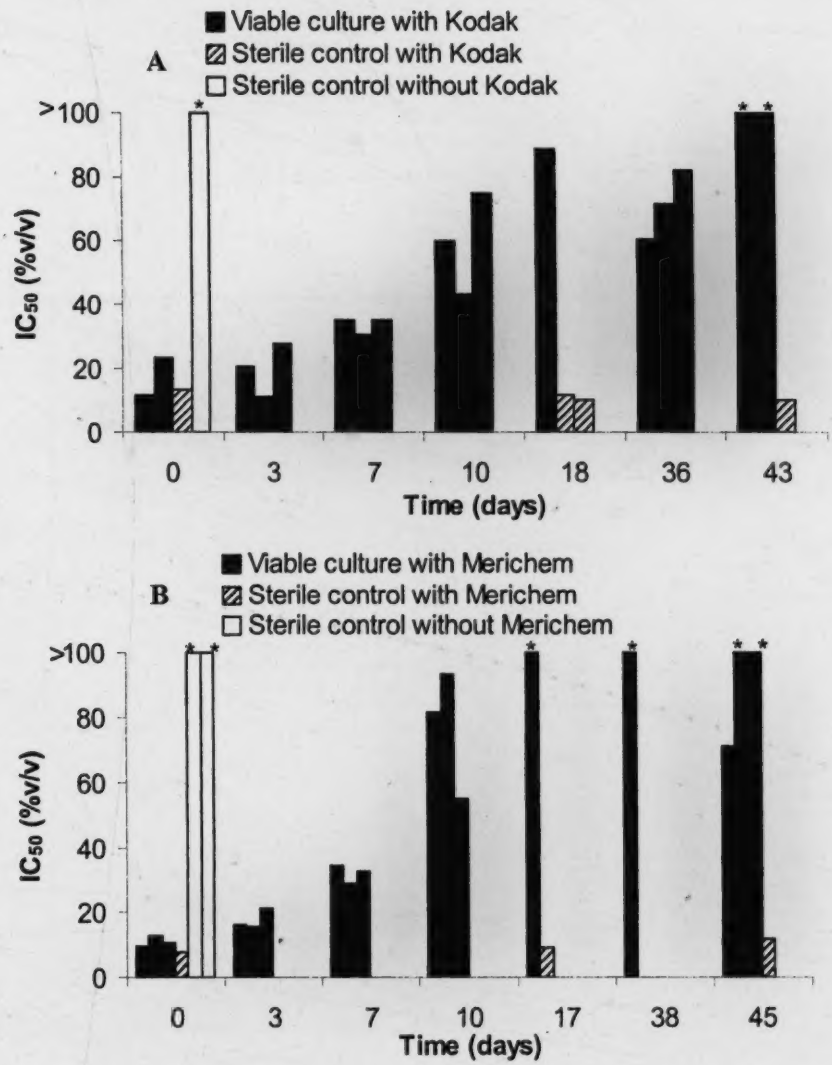

Figure 6.2 Acute toxicity results from 15-min Basic Microtox™ tests with supernatants of cultures that contained Kodak salts (A), and Merichem acids (B). The asterisks indicate cases when the results with the culture supernatant were indistinguishable from the Microtox™ reagent blank. At each time, the toxicity was determined on samples from 1 to 3 replicates, and each IC_{50} value is shown.

acids, had an acute toxicity similar to that of the day 0 viable enriched cultures that contained naphthenic acids. The second control contained heat-killed cells and all of the

other components of the culture medium, except the naphthenic acids. This solution showed no acute toxicity, with the IC_{50} value >100%. The toxicity of the supernatants from the viable enriched cultures decreased with incubation time, and by day 43, the IC_{50} values were >100%, which were indistinguishable from the Microtox™ reagent blank. In contrast, the toxicity of the sterile controls that contained naphthenic acids remained essentially constant over the 43-day incubation time, with IC_{50} values of 10% to 13% (v/v) (Figure 6.2A). By day 14, naphthenic acids in bottles with viable culture could not be detected by HPLC (Figure 6.1B), but the toxicity was not completely removed until day 43 (Figure 6.2A).

Culture supernatant samples were analyzed by GC-MS to determine the distribution of acids within the extracted naphthenic acids. The results from selected GC-MS analyses are shown as three-dimensional graphs in Figure 6.3. These plots show the relative abundance of ions with m/z values consistent with naphthenic acids (St. John et al. 1998, Holowenko et al. 2002). Any single bar in the three-dimensional graph represents the sum of m/z values for all those isomers with a given n and Z value. Initially, there was a wide array of acids in the naphthenic acids preparation extracted from the viable culture (Figure 6.3A). The most abundant ion was m/z=295. This corresponds to the major fragment ion of the *t*-butyldimethylsilyl derivative of naphthenic acids with n=15, Z=-4 (i.e. $C_{15}H_{26}O_2$), comprising 13% of all the naphthenic acids represented in the bar graph (Figure 6.3A). By day 18, there were virtually no residual naphthenic acids detected by the HPLC method (Figure 6.1) and there was a marked change in the fingerprint of the naphthenic acids (Figure 6.3B). In this sample, the three-dimensional graph was dominated by three acids, labeled "a", "b" and "c". These accounted for 14%, 18% and 9% of the ions detected in this extract. A similar fingerprint was observed by day 36 (Figure 6.3C), in which acids “a” and “b” accounted for 49% of the ions detected in this extract.

The total ion current (TIC) chromatogram of undegraded naphthenic acids appeared as a hump of unresolved compounds, with a few peaks protruding from the hump as observed in other studies (Herman et al. 1994, St. John et al. 1998, Jones et al. 2001). The TIC chromatogram of the extract from the culture that had incubated for 18 days showed a small hump with three distinct peaks corresponding to acids "a", "b",

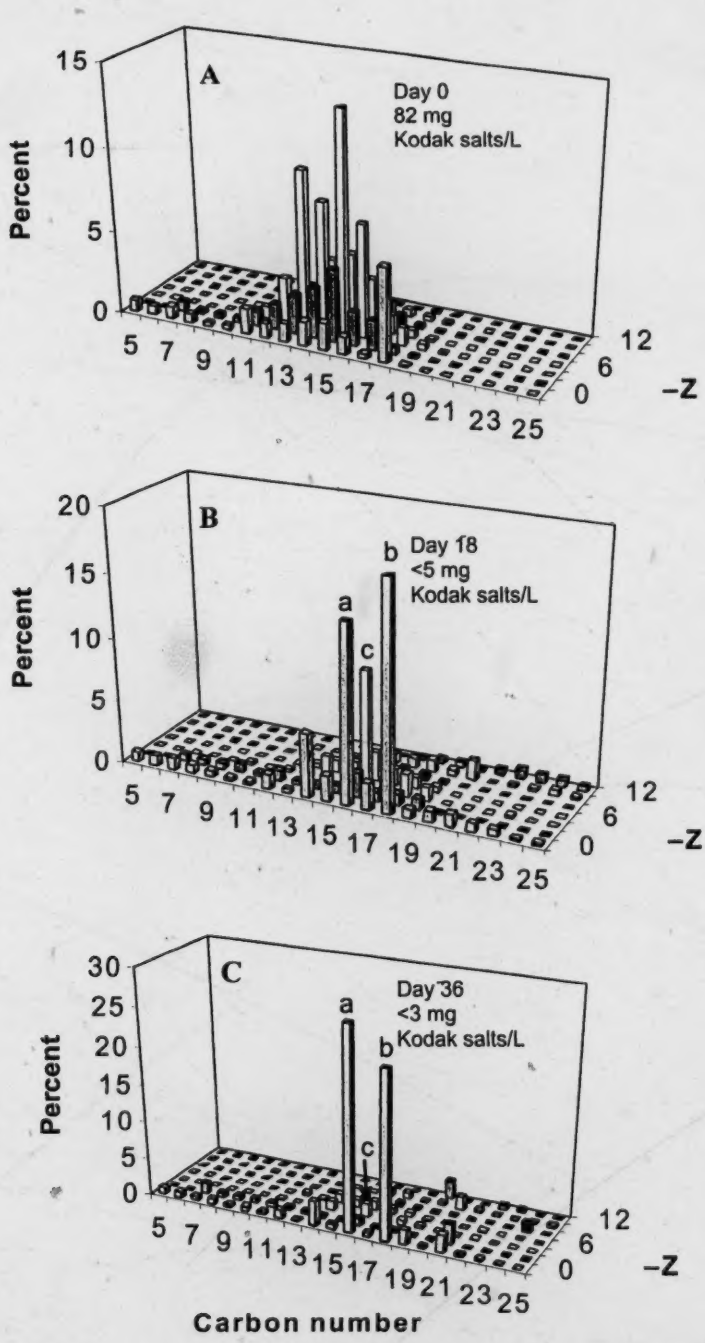

Figure 6.3 Changes in the distribution of residual acids recovered from the cultures degrading the Kodak salts. Results from GC-MS analyses on samples taken after 0 (A), 18 (B) and 36 days (C) of incubation. The sum of all the bars in each panel is 100%. Naphthenic acids concentrations are given for each time. Compounds "a", "b", and "c" are discussed in the text.

and "c". Acid "a" was a compound with n=16 and Z=0; acid "b" was a compound with n=18 and Z=0; and acid "c" was a compound with n=16 and Z=-4 (Figure 6.3B). The first two acids appeared to be palmitic and stearic acids, respectively. The *t*-butyldimethylsilyl derivatives of analytical grade palmitic and stearic acids were analyzed by GC-MS. They had the same retention times and mass spectra as acids "a" and "b", respectively, thus "a" and "b", were considered to be palmitic and stearic acids. However, there is the possibility that "a" and "b" were *t*-butyldimethylsilyl derivatives of two branched, acyclic acids that gave the same retention times and mass spectra as the *t*-butyldimethylsilyl derivatives of palmitic and stearic acids.

The mass spectrum of acid "c" appeared to be that of the di(*t*-butyldimethylsilyl) derivative of *o*-hydroxybenzoic (salicylic) acid. Derivatization of analytical grade salicylic acid (which has two reactive hydrogens) yielded a di(*t*-butyldimethylsilyl) derivative that had the same GC retention time and mass spectrum as acid "c". The calculated mass of this derivative is m/z 366. Characteristically, the *t*-butyldimethylsilyl derivatives lose a *t*-butyl group, yielding a base peak of $[M-57]^+$ (St. John et al. 1998). The loss of a *t*-butyl group from the di(*t*-butyldimethylsilyl) derivative of salicylic acid yields a base peak of m/z 309. High resolution mass spectrometry showed that this ion has an exact mass of 309.13432, consistent with the formula $C_{15}H_{25}O_3Si_2$ that would result from the loss of a *t*-butyl group from the di(*t*-butyldimethylsilyl) derivative of salicylic acid. Thus, the spectrum for acid "c" was consistent with salicylic acid. Based on the m/z value of 309, this compound was considered to be a naphthenic acid with n=16 and Z=-4 (Figure 6.3B). But salicylic acid is an aromatic acid, and does not fit the definition of a naphthenic acid proposed in the empirical formula. This result illustrates a limitation of this procedure of assigning compounds as naphthenic acids based only on fragment ions. This is discussed later in more detail.

To account for background dissolved carboxylic acids in the biodegradation experiments, a set of sterile controls was established that contained heat-killed cells from the enrichment culture that was grown on Kodak salts. No additional naphthenic acids were added to this control, which was extracted and analyzed by GC-MS. The resulting three-dimensional plot (not shown) had three major components that were acids "a", "b," and "c". In total, these three components accounted for 91% of the ions detected in this

extract. From this, it was evident that these three compounds were introduced with the inoculum from the enrichment culture.

Sterile controls, containing Kodak salts, were analyzed by GC-MS during this experiment. No major differences among the fingerprints of the naphthenic acids were observed (data not shown). Each of these three-dimensional graphs was essentially the same as that shown in Figure 6.3A. Consistent with these observation, the concentrations of naphthenic acids in the sterile controls were essentially constant (Figure 6.1A) and the toxicity of the sterile controls was unchanged over the incubation period (Figure 6.2A)

6.3.2 Biodegradation of the Merichem acids

The biodegradation of the Merichem acids yielded similar results to those shown in Figure 6.1A, except that degradation was apparent by day 3 in the cultures that contained the Merichem acids (Figure 6.1B). There was no loss of naphthenic acids from the sterile control, and the naphthenic acids concentrations in the enrichment culture microcosms decreased from an initial 110 mg/L to 8 mg/L by day 10 in the viable cultures (Figure 6.1B). The initial bacterial count was 5.6×10^4 cfu/mL. The count increased to 4.1×10^6 cfu/mL on day 3 and stayed constant over the remainder of the incubation. About 60% of the naphthenic acids carbon was detected as CO_2 after 17 days of incubation, and this value did not change during incubation up to 45 days.

The biodegradation of the Merichem acids reduced the toxicity of the culture supernatants (Figure 6.2B). At time zero, the IC_{50} values for the viable culture and naphthenic acids-containing sterile control were 10% and 8% (v/v), respectively. The IC_{50} values for the control that contained heat-killed cells without naphthenic acids were indistinguishable from that of the Microtox™ reagent blank (i.e. IC_{50} >100%). The toxicity of the sterile controls that contained naphthenic acids remained nearly constant (IC_{50} about 10% v/v) over the 45-day incubation time (as was seen for the Kodak salts in Figure 6.2A). However, the toxicity of the supernatant from the viable cultures decreased with incubation time, and on the last three sampling times (days 17, 38 and 45), no toxicity was detected in the viable culture supernatants, as was the case with the sterile controls devoid of naphthenic acids (Figure 6.2B).

Figure 6.4 shows the changes in the naphthenic acids fingerprints in the viable cultures during the first 10 days of incubation. The initial composition of the Merichem acids had a group of ions from n=9 to n=14 and Z=0 to Z=-4, with similar relative abundances between 3% and 8% (Figure 6.4A). By day 7, when the total naphthenic acids concentration had been reduced to 25 mg/L, the most abundant ions were those with Z=-4 (Figure 6.4B). The predominant ion (16%) corresponded to n=13 and Z=-4. Acids "a" and "c" (palmitic and salicylic acids, respectively) became more noticeable on day 7. By day 10, the naphthenic acids concentration had dropped to 8 mg/L and these were the two most abundant acids (Figure 6.4C), accounting for 68% of the detected ions. By day 45, when total naphthenic acids concentrations were reduced to <5 mg/L, acid "c" was the dominant component, making up 81% of the detected ions in the biodegraded Merichem acids. As with the Kodak salts, there were no major differences among the fingerprints of the Merichem acids in the sterile controls incubated for up to 45 days (data not shown). Each of these three-dimensional graphs was essentially the same as that shown in Figure 6.4A.

6.3.3 Statistical Analysis

Chapter 3 described a statistical method to compare the fingerprints of two naphthenic acids preparations, based on the carbon number distribution. As part of the method, the naphthenic acids were separated into three groups: (1) C5 to C13, (2) C14 to C21, and (3) the C22+ cluster. We used the same groupings, and summed the ion abundances in each GC-MS analysis of the extracted naphthenic acids from the cultures that degraded the Kodak salts or the Merichem acids. The changes in the distributions of the naphthenic acids with time are shown in Figures 6.3, 6.4 and 6.5.

Initially, 27% of the ions in the Kodak salts had masses that fell into group 1 naphthenic acids, and 73% of the ions fell into group 2 (Figure 6.5A). The proportion of ions in the first group declined, and by day 18, 11% of the ions fell into the range of group 1 naphthenic acids. Because the sum of all the entries in the three-dimensional graphs equals 100%, as the proportion of one group decreases, the proportion of another group must increase. On day 18, 83% of the ions fell in group 2, and 6% fell in group 3,

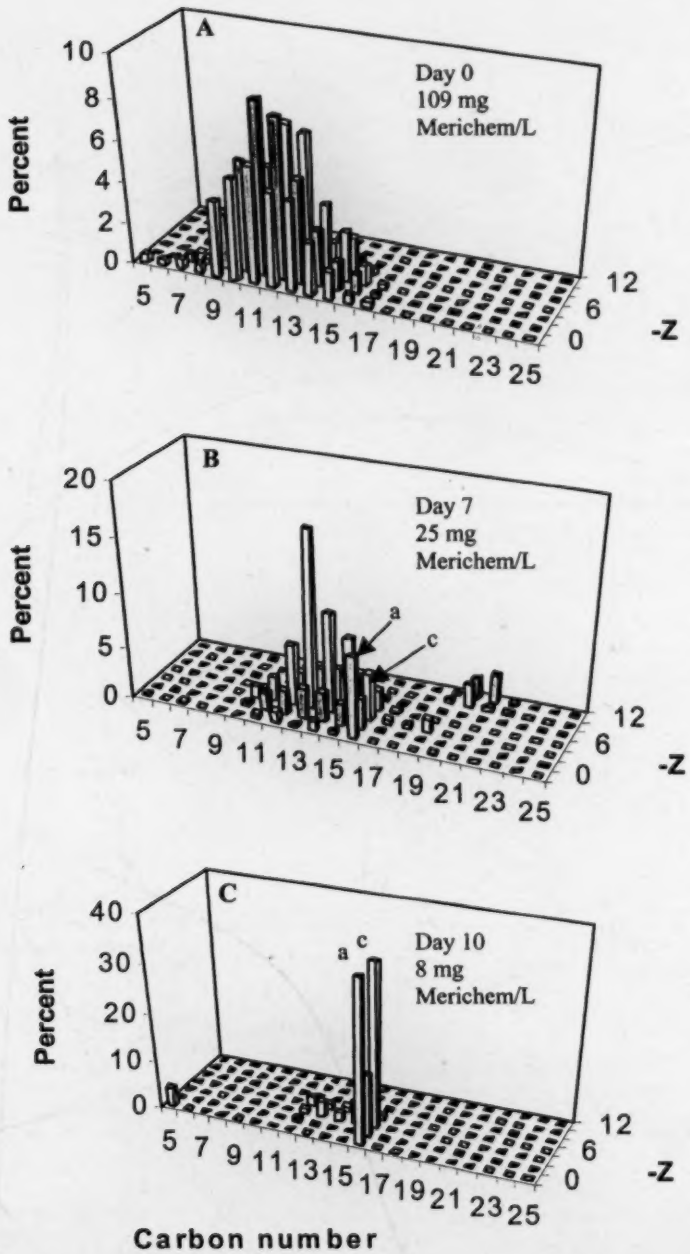

Figure 6.4 Changes in the distribution of residual acids recovered from the cultures degrading the Merichem acids. Results from GC-MS analyses on samples taken after 0 (A), 7 (B) and 10 days (C) of incubation. The sum of all the bars in each panel is 100%. Naphthenic acids concentration given for each time. Compounds "a" and "c" are discussed in the text.

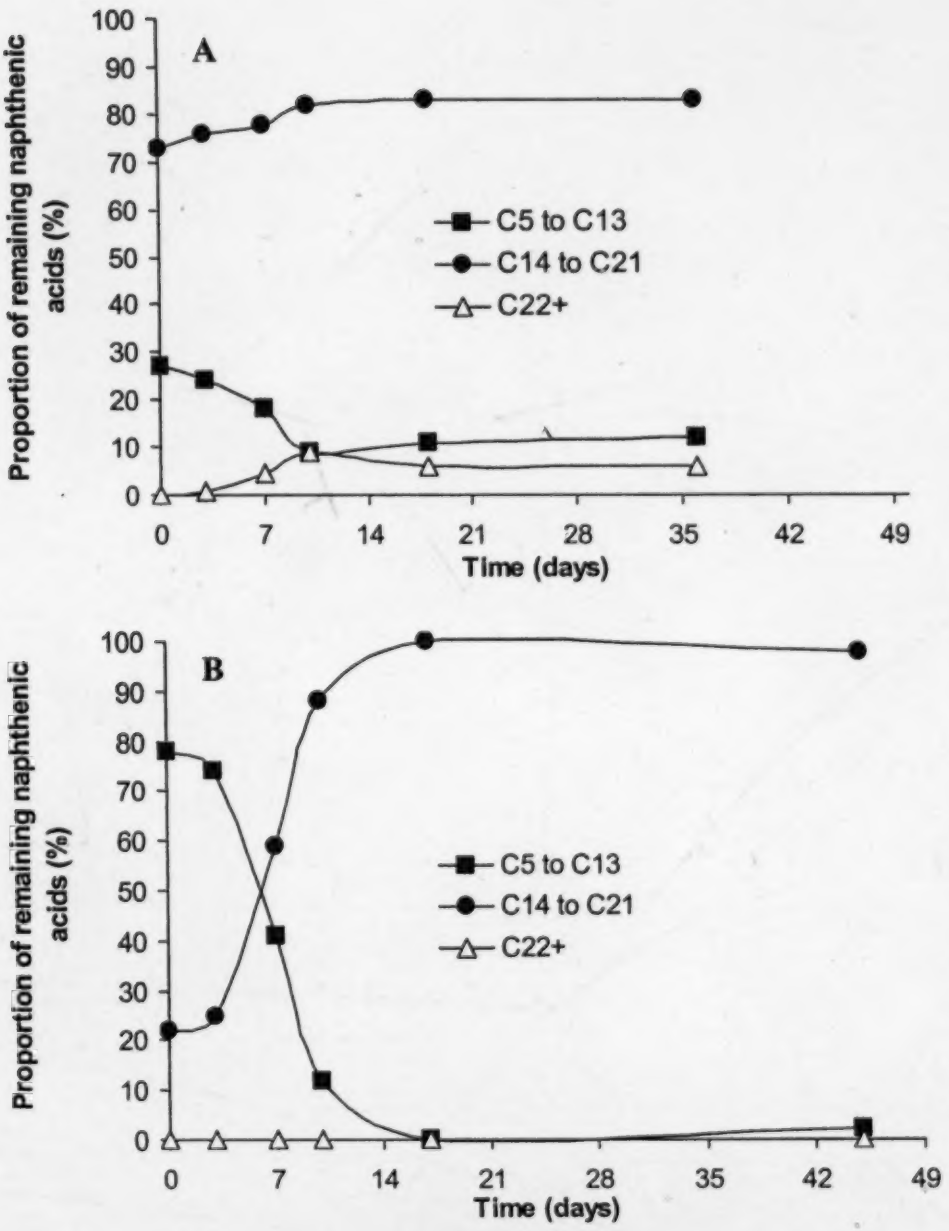

Figure 6.5 Changes in the relative abundances of naphthenic acids recovered from viable cultures after different incubation times. The proportions of ions were summed in groups according to carbon numbers (Chapter 3). Cultures were degrading the Kodak salts (A), or the Merichem acids (B).

the C22+ cluster. By day 36, ions were only found in group 2 (Figure 6.5A).

Examining the data from the biodegradation of the Merichem acids also showed the decrease in the low molecular weight acids (Figure 6.5B). Initially, 78% of the ions in this naphthenic acids preparation fell into group 1, and 22% fell into group 2. By day 7, 41% of the ions fell into group 1, and 59% of the ions fell into group 2. In addition. Figure 6.4B showed that compounds with Z=0 was preferentially biodegraded over those in the Z=-2 and Z=-4 families; while those in Z=-2 are preferentially biodegraded over those in the Z=-4 family. Examination of Figures 6.5A and 6.5B shows that nearly all of the naphthenic acids in the range of C5 to C10 were removed by day 7. Clearly these low molecular weight acids are the most susceptible to biodegradation. On day 17, all of the ions detected in the extracted Merichem acids fell into the second group (Figure 6.5B). The formation of biomass with palmitic and stearic acids (compounds "a" and "b") would contribute to the increase in the abundance of group 2 ions, observed in Figure 6.5.

6.4 Discussion

Laboratory studies have shown that the acute toxicity of solutions of commercial naphthenic acids (Herman et al. 1994) and of oil sands wastewaters containing naphthenic acids (Nix and Martin 1992, Herman et al. 1994, Lai et al. 1996, Moore et al. 2002) can be reduced by aerobic biodegradation. This work with Kodak salts and Merichem acids corroborate these findings. The Merichem acids were detoxified more quickly than Kodak salts. By day 17, the IC_{50} of the Merichem acids-degrading culture supernatant was >100% (v/v). This extent of detoxification of the Kodak salts was not observed until day 43 (Figure 6.2).

Because of its simplicity, the Microtox™ method has been used to monitor the decreases in acute toxicity of naphthenic acids solutions and oil sands wastewaters (MacKinnon and Boerger 1986, Herman et al. 1994, Lai et al. 1996). Studies have shown that waters with an IC_{50} >100% (v/v) (based on the 15-min Microtox™ test), have 96-h LC_{50} values of >100% in bioassays with *Daphnia magna* and trout (MacKinnon and Boerger 1986, Schramm et al. 2000).

None of the previous biodegradation studies have provided detailed information on the specific changes to the naphthenic acids concentrations and to the relative compositions of the naphthenic acids over the course of the incubations. The development of an HPLC method for naphthenic acids quantification and the application of the GC-MS method have allowed me to provide greater insight into the biodegradation of two commercial naphthenic acids preparations. Figure 6.1 illustrates the decrease in naphthenic acids concentrations approaching zero by day 14. Figures 6.3, 6.4 and 6.5 show the dramatic changes in the fingerprint of the extracted naphthenic acids.

The computer program used to analyze the GC-MS data handles ion masses up to nearly 550 (carbon number 33 with ionized dimethylsilyl fragment) (Holowenko et al. 2002, St. John et al. 1998) and the three-dimensional graphs usually include carbon numbers up to 33 (Holowenko et al. 2002, Chapter 3). It is common for the naphthenic acids from the oil sands and petroleum to have these high molecular weight components (Fan 1991, Holowenko et al. 2002, Morales-Izquierdo 1999, Hsu et al. 2000). However, commercial preparations of naphthenic acids typically have a narrower range of molecular weights based on GC-MS analyses (St. John et al. 1998, Chapter 3) or other mass spectrometry methods (Morales-Izquierdo 1999, Hsu et al. 2000, Jones et al. 2001). The majority of the acids in the commercial preparations have molecular weights below about 350 (carbon number <23). Thus, the three-dimensional plots in Figures 6.4 and 6.5 were truncated at carbon number 25.

Herman et al. (1994) also measured the mineralization of the Kodak salts after 25 days incubation, they observed that 48% of the organic carbon was oxidized to CO_2. Using a different enrichment culture and a longer incubation time, I observed about 60% of the organic carbon in the Kodak salts was mineralized to CO_2 (Figure 6.1A). These findings are in agreement with the general assumption that aerobic heterotrophic activity releases about 50% of the substrate carbon as CO_2 (Maier et al. 2000). The three-dimensional plots in Figure 6.3 provide the first evidence that the composition of naphthenic acids changes during mineralization in laboratory cultures.

After 18 days incubation, the concentration of naphthenic acids in the cultures that contained Kodak salts was reduced by >90% (Figure 6.1A), and the resulting GC-MS fingerprint of the residual naphthenic acids in the Kodak salts was dominated by palmitic,

stearic and salicylic acids. Palmitic acid is the predominant fatty acid in Gram-negative bacteria, which also contain stearic acid (Lechevalier and Lechevalier 1988). These fatty acids are also found in Gram-positive bacteria (O'Leary and Wilkinson 1988) and in yeasts (Lechevalier and Lechevalier 1988). Thus, the appearance of these acids in Figures 6.3 and 6.4 is likely the result of microbial growth on the naphthenic acids. Palmitic and stearic acids are readily biodegradable, and small amounts of these two acids were detected in the TIC chromatograms of the extracts from the final sampling times, days 43 and 45, for both naphthenic acids preparations. However, their abundances were so low that they did not reach the 1% "minimum occurrence" value required to be averaged by the software used to handle the data from the mass spectrometer. Therefore, palmitic and stearic acids did not appear in the three-dimensional plots from the last sampling times of the biodegradation experiments (data not shown).

The origin of the salicylic acid in the cultures is unknown. Serum bottles with sterile medium, sterile medium with heat-killed cells and sterile medium in storage containers were extracted with dichloromethane and these extracts were analyzed by GC-MS to determine if the salicylic acid was a contaminant. Some extracts showed traces of palmitic and stearic acids, but no salicylic acid was found. Salicylic acid might be a minor component of the naphthenic acids because aromatic compounds have been detected in naphthenic acids from crude oils (Seifert and Teeter 1970, Hsu et al. 2000). Salicylic acid is an intermediate in the metabolism of 3-cyclohexene carboxylic acid by *Alcaligenes faecalis* (Blakley and Papish 1982). *p*-Hydroxybenzoic acid, an isomer of salicylic acid, is an intermediate in the biodegradation of cyclohexanecarboxylic acid by *Alcaligenes* strain W (Taylor and Trudgill 1978). Thus, some microorganisms in my undefined mixed cultures may have transformed one of the carboxylic acids in the naphthenic acids to salicylic acid. It is surprising that salicylic acid remained in the cultures because it is a transient intermediate in the metabolism of hydrocarbons such as naphthalene, phenanthrene, and anthracene (Gibson and Subramanian 1984). Although salicylic acid was the dominant compound found in the residual extracted naphthenic acids (e.g. Figure 6.4C), HPLC showed that by the end of the batch culture experiments,

the concentrations of naphthenic acids were very low. No attempt was made to quantify the salicylic acid.

Based on examinations of oil sands process-affected waters with various ages, Holowenko et al. (2002) suggested that biodegradation of lower molecular weight naphthenic acids would occur preferentially, shifting the proportion of remaining naphthenic acids to the higher molecular weight range. Data from the biodegradation of the Kodak salts and Merichem acids (Figure 6.5) support this hypothesis.

Figure 6.5A shows the appearance of ions in the C22+ cluster in the extracted naphthenic acids from the Kodak salts-degrading cultures. On day 10, 9% of the total ions fell into this group. Similarly, the proportion of these ions detected in the Merichem acids-degrading culture was small (<1% on day 7). Initially, there were no ions detected in this group (Figures 6.4A and 6.5A), but it is possible that the biodegradation of the lower molecular weight acids simply increased the relative proportion of the C22+ cluster of naphthenic acids so they were detectable.

However, Figure 6.6 suggests a means by which a simple microbial oxidation of a naphthenic acid (C15, Z=-4) may lead to the formation of a derivatized metabolite that falls into the C22+ cluster. Without microbial oxidation, Scheme 1 shows the product of derivation and the structure of the major fragment (the base peak) detected by the mass spectrometer. The m/z 295 ion is assigned as a naphthenic acid, with carbon number 15 and Z=-4 (St. John et al. 1998).

The first step in microbial metabolism of a cycloalkane is often hydroxylation (Neilson 1999). Scheme 2 (Figure 6.6) shows a hydroxylated naphthenic acid that might be formed by microbial oxidation. This would introduce a second reactive hydrogen that would react with the derivatizing agent yielding a di(*t*-butyldimethylsilyl) derivative. Fragmentation in the mass spectrometer would yield the base peak with m/z 425. This ion would be assigned as a naphthenic acid with carbon number 24 and Z=0 (St. John et al. 1998). Alternatively, a common means of microbial oxidation of an alkyl side chain is through terminal oxidation yielding a carboxylic acid (Perry 1984, Trudgill 1984). If the butyl side chain of the naphthenic acid, shown in Figure 6.6, was oxidized in this manner to yield a dicarboxylic acid, derivatization and fragmentation in the mass spectrometer would yield a base peak of m/z 439 ($[C_{23}H_{43}Si_2O_4]^{\cdot +}$). This would be assigned as a

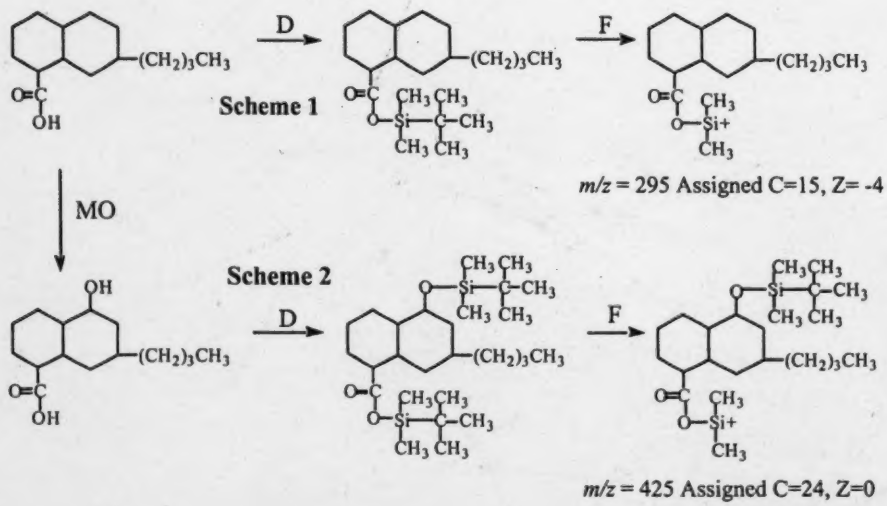

Figure 6.6 Hypothetical formation of a C22+ ion from a naphthenic acid with carbon number 15 and Z=0. With no microbial oxidation, Scheme 1 shows the product of derivatization (D), and the major ion after fragmentation (F) in the mass spectrometer. Scheme 2 shows a possible product of microbial oxidation (MO), and the product of derivatization (D), and the major ion after fragmentation (F) in the mass spectrometer.

naphthenic acid with carbon number 25 and Z=0 (St. John et al. 1998). Thus, these simple microbial transformations would yield intermediates (or products) that will appear in the C22+ cluster.

These two examples illustrate that the assignments of ions, based solely on their m/z values, may lead to erroneous results. That is, a biotransformation product, like the one shown in Scheme 2 (Figure 6.6), may be considered a higher molecular weight naphthenic acid, or as discussed above, salicylic acid (acid "c") may be incorrectly considered to be a naphthenic acid with n=16 and Z=-4. However, virtually all mass spectral analyses of naphthenic acids rely solely on assigning n and Z numbers based on ions with pre-selected masses (Dzidic et al. 1988, Fan 1991, St. John et al. 1998, Hsu et al. 2000, Rudzinski et al. 2002). Currently, there is no method available to separate the individual compounds in the complex naphthenic acids mixtures, and until better separation and analytical methods are developed, the ambiguous assignments of ions to particular n and Z numbers will continue.

From the GC-MS analysis of *t*-butyldimethylsilyl derivatives of a mixture of carboxylic acid, most fragment ions with odd m/z values between 173 and 551, inclusive, are assigned to be naphthenic acids (St. John et al. 1998). Holowenko et al. (2002) outlined some restriction on the m/z values that are assigned to be naphthenic acids, leaving a total of 156 ions with n ranging from 5 to 33 and Z ranging from 0 to –12, inclusive. Remembering that each ion with an odd m/z value could be the sum of the base peaks of many different isomers, the actual number of compounds in a naphthenic acids preparation could be enormous. Despite some shortcomings discussed above, the application of this GC-MS method and the study of the changes in the three-dimensional fingerprints of extracts from active cultures have provided unprecedented insights into the order in which naphthenic acids are biodegraded and into the changes in the distribution of the residual extractable acids during the biodegradation of these commercial naphthenic acids. These results have also demonstrated that toxicity decreases as the composition of the residual naphthenic acids changes. It is not known whether toxicity reduction is simply because biodegradation decreases the overall concentration of naphthenic acids or if microbial metabolism preferentially removes the more toxic naphthenic acids.

This work showed that two commercial naphthenic acids preparations can be biodegraded extensively under laboratory conditions, using aerated cultures with an abundant supply of inorganic nutrients including phosphorus and fixed nitrogen. The rates of biodegradation of commercial naphthenic acids in the environment will be much slower. Based on previous GC-MS analyses, the compositions of the naphthenic acids in the oil sands tailings ponds are more complex than those of commercial preparations (Holowenko et al. 2002). Specifically, naphthenic acids extracted from the tailings ponds contain compounds in the C22+ cluster, which are essentially absent in the commercial preparations. Future studies will apply the methods used in this chapter to follow the biodegradation of the naphthenic acids from the MLSBF.

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7. LESSONS LEARNED FOR INTERPRETING *t*-BUTYLDIMETHYLSILYL EI GC-MS RESULTS

7.1 Introduction

Chemically and structurally related carboxylic compounds with the general formula $C_nH_{2n+Z}O_2$ are called naphthenic acids. These compounds may be cyclic or acyclic, where acyclic components are highly branched (Rudzinski et al. 2002). An alkyl group is also attached to cyclic naphthenic acids depicted as "R" in Chapter 1, Figure 1.1. The smallest expected cyclic structure would be a cyclopentane moiety substituted with a methyl and a carboxylic acid; the largest compound is expected to have a molecular weight of less than 1000 Da (CONRAD 1998). The carboxylic acid group is generally not directly fused to the ring (Brient et al. 1995, Holowenko et al. 2002).

Crude oils naturally contain naphthenic acids (Seifert and Teeter 1969, Seifert et al. 1969, Fan 1991, Tomczyk et al. 2001). Egyptian crudes have low naphthenic acids concentrations at 0.0015%, whereas those from the Athabasca oil sands contain about 2% (w/w) naphthenic acids (CONRAD 1998). High carboxylic acid concentrations are the result of having either an immature deposit, or bacterially degraded petroleum (Tissot and Welte 1978). Naphthenic acids in crude oil cause processing equipment corrosion. This occurs in areas of high velocity, in combination with temperatures between 220 °C to 400 °C (Turnbull et al. 1998, Kane and Cayard 1999).

The naphthenic acids in the Athabasca oil sands are released during the hot caustic extraction process used to extract bitumen (Schramm et al. 2000). The resulting extraction process wastewaters are stored in artificial ponds, such as the Mildred Lake Settling basin, which was created in 1978 (MacKinnon 1989). Naphthenic acids are commercially produced by extracting the 200 °C to 370 °C distillate, using 2% to 10% NaOH (Brient et al. 1995). Their uses include: wood preservation, vinyl resin stabilization, and emulsification (Brient et al. 1995). Commercially used naphthenic acids, as well as those in storage ponds, are problematic because of their toxicity. Naphthenic acids were determined to be the most toxic components of tailings water

(Madill et al. 2001). They are toxic to plants (Kamaluddin and Zwiazek 2002), fish (Dokholyan and Magomedov 1984), and mammals (Khanna et al. 1971, Rogers et al. 2002b). They have also been found to be the toxic components in petroleum refinery effluents (Wong et al. 1996).

Structure is related to the corrosivity (Turnbull et al. 1998), and possibly toxicity (Holowenko et al. 2002) of naphthenic acids. Mass spectrometry (MS) has been used as a means of determining the structural composition of naphthenic acids. Ionization methods include electron impact (Seifert and Teeter 1969, Seifert et al. 1969, Lebedevskaya et al. 1978, Green et al. 1994, St. John et al. 1998, Tomczyk et al. 2001), fast atom bombardment (Fan 1991), chemical ionization (Dzidic et al. 1988, Hsu et al. 2000), and electrospray ionization (Qian et al. 2001, Rogers et al. 2002a, Rudzinski et al. 2002, Barrow et al. 2003, Gabryelski and Froese 2003, Lo et al. 2003).

Of all the MS methods so far developed for naphthenic acids analysis, *N*-methyl-*N*-(*t*-butyldimethylsilyl)trifluoroacetamide (MTBSTFA) derivatization with electron impact MS is the most accessible (St. John et al. 1998). Holowenko et al. (2002) applied this method to the analysis of Athabasca oil sands tailings ponds. The observed masses can be organized into a two-dimensional matrix, where each cell corresponds to a carbon number, Z family combination (St. John et al. 1998, Holowenko et al. 2002). Holowenko et al. (2002) took a similar approach as St. John et al. (1998), but only considered ions that followed the general definition of naphthenic acids. The relative intensities of these ions were then summarized into three-dimensional graphs as a means of better illustrating the differences in ion distributions when both carbon number and Z families were considered simultaneously. Variations of three-dimensional graphs representing naphthenic acids component distributions were also used by Tomczyk et al. (2001), and Rudzinski et al. (2002).

The derivatizing agent produces *t*-butyldimethylsilyl naphthenic acid esters, which are ionized to dimethylsilyl esters (St. John et al. 1998). It has been noted that the ^{30}Si isotope might affect the proportion of observed ions of compounds with +2 Z value to that of a very abundant ion (St. John et al. 1998). Although MTBSTFA react with acidic alcohols, St. John et al. (1998) found that structurally hindering access of the derivatizing agent to this group inhibits the reaction. St. John et al. (1998) have

determined that this method provides the same information as fluoride ion chemical ionization. This study attempts to determine the effects of stable isotopes. This was done by utilizing the theoretical abundance (International Union of Pure and Applied Chemistry 1991) of stable isotopes of atoms found in the dimethylsilyl ester of naphthenic acids. Six surrogate naphthenic acids were analyzed individually to determine the effects of stable isotopes and other fragmentation events on the parent ion. Mixtures of these six compounds were also used to determine the efficiency of the derivatization reaction, ionization and detection.

7.2 Materials and Methods

7.2.1 Naphthenic acids used for GC-MS studies

Four naphthenic acids extracts were used for these studies. They were: MLSB7, which was extracted by Holowenko in 2002 from experimental ponds containing naphthenic acids, which were undisturbed for seven years. Ore extracts from Syncrude, which were also used in Chapter 4. These were: Syncrude B (Basal ore, good processing), Syncrude E (Syncrude Aurora transition ore), Syncrude F (Syncrude North mine upper bench). These samples were chosen because they contained compounds in group 3, as described in Chapter 3.

7.2.2 Naphthenic acids derivatization and GC-MS

Naphthenic acids were derivatized as outlined by St. John et al. (1998) using MTBSTFA which contained 1% *t*-butyldimethylsilyl chloride (Sigma, St. Louis, MO). A modification of the method was that the reaction solution was dried under N_2 and reconstituted to the original volume (usually 100 μL) with dichloromethane. The GC-MS method was described by Holowenko et al. (2002). Spectral data were acquired using the Mass Spec Data System for Windows version 14.0c (Mass Spec Services, England). Peak ion intensity values were averaged over the elution of the naphthenic acids hump, generally from retention time 10 min onward. The "minimum occurrence" variable for

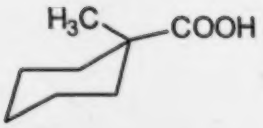
the averaged data was set at 1%, which meant that the peak ion had to occur in at least 1% of the total scans averaged to be included in the final average data outputted from the computer. This was performed on all the data, except where it was indicated that 0% minimum occurrence was chosen. In the case where 0% was chosen, all of the scanned ions were included in the average. The averaged peak intensity values were entered into a Microsoft Excel spreadsheet, which selected only those masses that corresponded to derivatized naphthenic acids with carbon numbers 5 to 33 and Z values 0 to -12.

7.2.3 Evaluation of the GC-MS method using model naphthenic acids

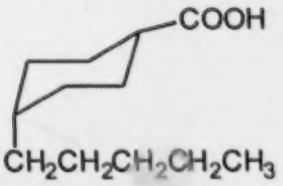
The following six pure compounds (Figure 7.1) were derivatized with MTBSTFA: 1-methyl-1-cyclohexanecarboxylic acid, *trans*-4-pentylcyclohexane-carboxylic acid, 2-hexyldecanoic acid (Aldrich Chemical Co, Milwaukee, WI), eicosanoic acid (Applied Sciences Laboratories Inc., State College, PA), dicyclohexylacetic acid (Aldrich), and 5β-cholanic acid (Sigma Chemicals Co., St. Louis, MO). The molar concentrations of all six compounds were calculated from the measured mass and volume used in analyses where a mixture of the six compounds was analyzed. These were derivatized and analyzed by GC-MS individually and in mixtures of all six surrogate naphthenic acids. The resulting average mass spectra were studied to determine the influence of the other naturally occurring heavy isotopes of carbon (^{13}C), and silicon (^{29}Si and ^{30}Si) on the three-dimensional plots generated to summarize the distribution of naphthenic acids in a mixture.

High resolution MS analysis of 5β-cholanic acid was performed using a Kratos MS50 mass spectrometer (Manchester, UK) by personnel in the Mass Spectrometry Laboratory (Chemistry Department, University of Alberta). The samples were introduced to the MS using a probe.

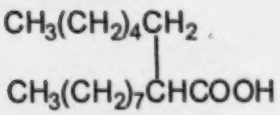
GC (HP 6890) coupled to an atomic emission detector (AED) was used to evaluate any effects that the MS may have on ion detection. The same samples used for GC-MS analysis were submitted for GC-AED analysis at Syncrude Research Centre (Edmonton, Alberta). This was performed using a temperature gradient of 10 °C/min, with an initial temperature of 40 °C and a final temperature of 300 °C, and a 25 m methyl



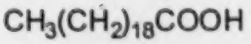
1-Methyl-1-cyclohexane carboxylic acid



trans-4-Pentylcyclohexane carboxylic acid

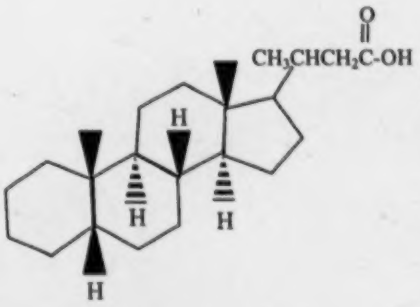


2-Hexyldecanoic acid



Eicosanoic acid

Dicyclohexylacetic acid



5β-Cholanic acid

Figure 7.1 Structures of surrogate naphthenic acid compounds used to determine the need for isotope correction.

siloxane capillary column. Total run time was 61 min. The transfer line was held at 250 °C, and 1.5 μL of the sample was injected using the split injection mode. Helium was used as the carrier gas. Atomic emission of silicon was detected at 251.6 nm as in Kala et al. (1997). Since this is specific to detecting Si atoms, the magnitude of the signals for all six compounds were expected to depend on their concentrations, and efficiency of the reaction.

7.2.4 *Statistical analysis*

Details of the statistical method used to compare the results from GC-MS are given in Chapter 3. Briefly, the data in each matrix were divided into three groups: Group 1 = carbon numbers 5 to 13, inclusive; Group 2 = carbon numbers 14 to 21, inclusive, and group 3 = carbon numbers 22 to 33, inclusive. The members of group 3 were chosen to match the C22+ cluster that was observed by Holowenko et al. (2002).

Each percent value in the matrix was divided by 100, and the arcsine of each quotient was taken as a variance stabilizing transformation. The arcsine-transformed data for each group from one naphthenic acid sample was compared with the corresponding arcsine-transformed data of the corresponding group from a second naphthenic acid sample by applying an independent two-sample t-test assuming equal variance. The groups were considered to be different if the P value for the two-tailed test was <0.05.

7.2.5 *Correction for stable isotopes*

The theoretical ion intensity (I_T), is the sum of the observed ion intensity (I_M), the intensity of the A+1 ion (I_{A+1}), and the intensity of the A+2 ion (I_{A+2}). Ion distributions determined using the magnetic sector instrument, under low MS resolution were corrected for stable isotopes using the following equation:

$$I_T = I_M + I_{A+1} + I_{A+2}$$

where:

I_T = total ion intensity

I_M = observed ion intensity

I_{A+1} = intensity of the A+1 ion

I_{A+2} = intensity of the A+2 ion

Equation 7.1

The A+1 ion occurs when there is one ^{13}C atom or a ^{29}Si atom. The A+2 ion occurs when

there are two ^{13}C atoms, a combination of one ^{13}C and one ^{29}Si atom, or having one ^{30}Si atom. Using isotopic abundance from International Union of Pure and Applied Chemistry (1991), the probabilities can therefore be expressed as:

$$P_{A+1} = (0.0107 \times C_m) + (0.0471)$$

Equation 7.2

$$P_{A+2} = (0.0107 \times C_m)^2 + (0.031) + (0.047 \times 0.0107 \times C_m)$$

Equation 7.3

where:

P_{A+1} is the likelihood of getting the A+1 ion

P_{A+2} is the likelihood of getting the A+2 ion

C_m is the number of carbon atoms in the compound including the additional two carbons from the dimethylsilyl moiety in the derivatized naphthenic acid

As shown by Equation 7.2, the probability of getting an A+1 ion is equal to the abundance of the ^{13}C atom (1.07%), multiplied by the total number of carbon atoms in the ion (since each carbon in the compound has a 0.0107 chance of being ^{13}C), plus the abundance of ^{29}Si (since there is expected to be only one Si in a derivatized naphthenic acid). From Equation 7.3, the likelihood of getting an A+2 ion is the sum of all the events that could produce this ion. The expression $(0.0107 \times C_m)^2$ takes into account the contribution of two ^{13}C atoms, while the expression $(0.047 \times 0.0107 \times C_m)$ takes into account the contribution of a ^{13}C and a ^{29}Si (4.7% abundance). The multiplier is used, because the likelihood of getting an A+2 ion depends on two events occurring simultaneously. To those terms is added the abundance of the ^{30}Si atom (3.1%). Equation 7.1 then becomes:

$$I_T = I_M + [(0.0107 \times C_m) + (0.0471)]I_T + [(0.0107 \times C_m)^2 + (0.031) + (0.047 \times 0.0107 \times C_m)]I_T$$

Equation 7.4

Equation 7.4 shows that the intensity of the A+1 and A+2 ions are equivalent to the likelihood of those ions occurring, multiplied by the theoretical total intensity. However, the observed ion intensity (I_M) is the sum of the intensity of the A ion, as well as the A+2 of an ion, with the same carbon number, but one less ring (i.e. A-2). This term, must therefore be corrected as follows:

$$I_M = I_A - [(0.0107 \times C_m)^2 + (0.031) + (0.047 \times 0.0107 \times C_m)]I_{A-2}$$

Equation 7.5

where:

I_A is the intensity of the A ion (observed from MS)

I_{A-2} is the intensity of the A-2 ion (calculated)

Simplifying and rearranging Equation 7.1, after substituting in Equations 7.2 through 7.5 results in Equation 7.6:

$$I_T = \frac{I_A - [(0.0107 \times C_m)^2 + (0.031) + (0.047 \times 0.0107 \times C_m)]I_{A-2}}{[0.922 - (0.0107 \times C_m)^2 - (0.0102 \times C_m)]}$$

Equation 7.6

Equation 7.6 is used to correct the ions observed in the three-dimensional graphs. Corrections were done only on samples discussed in Section 7.3.2.

7.3 Results and Discussion

7.3.1 Ions in three-dimensional plots originating from stable isotopes, and a minor fragmentation event

Six surrogate naphthenic acids, shown in Figure 7.1 were used to determine the effects of stable isotopes on the intensities of ions observed in the three-dimensional plots

produced after GC-MS analysis. Figure 7.2A shows the three-dimensional plot of 5β-cholanic acid after GC-MS analysis. Four ions were considered naphthenic acids, and plotted in the three-dimensional graph. These had m/z values of 281, 417, 419, and 459(Table 7.1 bold). Three out of the four signals were from 5β-cholanic acid; m/z 281 (C14, Z=-14) was determined to come from the GC column bleed. This ion was present in solvent injections and at time points other than when the sample elutes. As shown in Figure 7.2B, two of the ions (m/z = 417, and 459) were from different fragmentations of

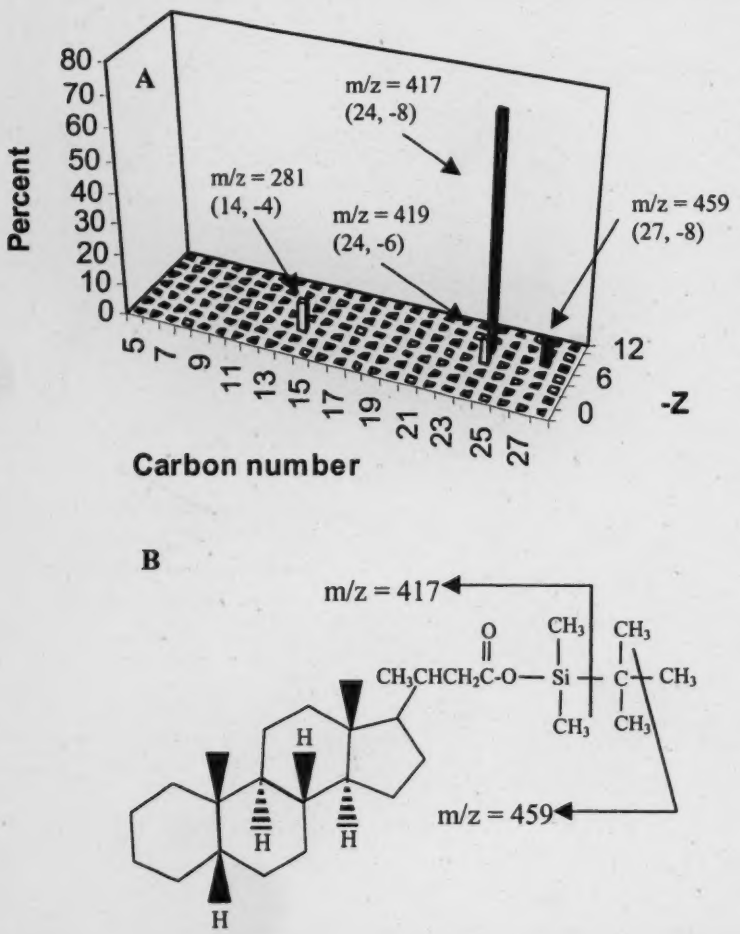


Figure 7.2 Three-dimensional plot produced from the GC-MS analysis of the *t*-butyldimethylsilyl derivative of 5β-cholanic acid (A), and the fragmentation of the derivative, where the m/z values are given for the naturally most abundant isotopes of C, H, and Si (B).

the *t*-butyldimethylsilyl derivative. Although break-up of the *t*-butyl group, to give the $[M+57]^+$ ion, was the major fragmentation event (m/z = 417), a methyl from the *t*-butyl group can also breakup to give an $[M+99]^+$ ion (m/z = 459). On the other hand, A+2 ions (A, the major ion, plus 2 Da) are formed because of the contributions of ^{13}C and ^{30}Si isotopes after the break up of the *t*-butyl group. These were verified using high resolution MS. These extra ions are included in the three-dimensional graphs for most compounds.

The percent contribution of the A+2 and $[M+99]^+$ ions to the three-dimensional plots were determined for the six surrogate naphthenic acids shown in Figure 7.1. These observations are summarized in Table 7.2. All of these extra ions are considered naphthenic acids (Table 7.1 bold), except for the A+2 ion of 2-hexyldecanoic acid, which has an m/z of 315. This ion is not considered a naphthenic acid since it violates the naphthenic acids definition and is therefore ignored by the Excel program. As shown in Table 7.2, these extra ions make up a small percentage of the ions in the three-dimensional graph, with a minimum of 0% for the $[M+99]^+$ ion of dicyclohexylacetic acid and a maximum of 10% for the A+2 ion of 2-hexyldecanoic acid. The sum of A, A+2 and $[M+99]^+$ ions may be less than 100% because other ions, not considered in this table, may be present.

7.3.2 Correction for stable isotopes

Table 7.3 summarizes the absolute and relative intensities of the A and A+1 ions (A, the major ion, plus 1 Da) of the six pure compounds used in this study. The last column of Table 7.2 shows that a significant proportion of the $[M+57]^+$ ions from each compound appear as A+1 ions because of the presence of isotopes such as ^{13}C and ^{29}Si. As expected, the compounds with the greatest number of carbon atoms (eicosanoic and 5β-cholanic acids) have the highest relative abundance of the A+1 ion.

As shown in Tables 7.2 and 7.3, stable isotopes decreases the observed ion intensity of ions considered as naphthenic acids. This effect becomes more serious as molecular weight increases. Because of this, corrections for stable isotopes (^{13}C, ^{29}Si, and ^{30}Si) were investigated. St. John et al. (1998) have shown that fragmentation of the *t*-butyl group is the primary event in the ionization of *t*-butyldimethylsilyl derivatives. As

Table 7.1 Expected carbon number (n) and Z family, based on nominal weight observed by MS of *t*-butyldimethylsilyl derivatives, given the formula $C_nH_{2n+Z}O_2$ and the definition of a naphthenic acid. Crossed cells represent compounds not considered naphthenic acids.

Carbon number	Z Family						
	0	-2	-4	-6	-8	-10	-12
5	$C_5H_{10}O_2$[a] 159[b]	~~$C_5H_8O_2$ 157~~	~~$C_5H_6O_2$ 155~~	~~$C_5H_4O_2$ 153~~	~~$C_5H_2O_2$ 151~~	~~C_5O_2 149~~	---
6	$C_6H_{12}O_2$ 173	~~$C_6H_{10}O_2$ 171~~	~~$C_6H_8O_2$ 170~~	~~$C_6H_6O_2$ 168~~	~~$C_6H_4O_2$ 166~~	~~$C_6H_2O_2$ 164~~	~~C_6O_2 162~~
7	$C_7H_{14}O_2$ 187	$C_7H_{12}O_2$ 185	~~$C_7H_{10}O_2$ 183~~	~~$C_7H_8O_2$ 181~~	~~$C_7H_6O_2$ 179~~	~~$C_7H_4O_2$ 177~~	~~$C_7H_2O_2$ 175~~
8	**$C_8H_{16}O_2$**[c] **201**	**$C_8H_{14}O_2$ 1 199**	~~$C_8H_{12}O_2$ 197~~	~~$C_8H_{10}O_2$ 195~~	~~$C_8H_8O_2$ 193~~	~~$C_8H_6O_2$ 191~~	~~$C_8H_4O_2$ 189~~
9	$C_9H_{18}O_2$ 215	$C_9H_{16}O_2$ 213	~~$C_9H_{14}O_2$ 211~~	~~$C_9H_{12}O_2$ 209~~	~~$C_9H_{10}O_2$ 207~~	~~$C_9H_8O_2$ 205~~	~~$C_9H_6O_2$ 203~~
10	$C_{10}H_{20}O_2$ 229	$C_{10}H_{18}O_2$ 227	$C_{10}H_{16}O_2$ 225	~~$C_{10}H_{14}O_2$ 223~~	~~$C_{10}H_{12}O_2$ 221~~	~~$C_{10}H_{10}O_2$ 219~~	~~$C_{10}H_8O_2$ 217~~
11	$C_{11}H_{22}O_2$ 243	**$C_{11}H_{20}O_2$ 241**	$C_{11}H_{18}O_2$ 239	~~$C_{11}H_{16}O_2$ 237~~	~~$C_{11}H_{14}O_2$ 235~~	~~$C_{11}H_{12}O_2$ 233~~	~~$C_{11}H_{10}O_2$ 231~~
12	**$C_{12}H_{24}O_2$ 257**	**$C_{12}H_{22}O_2$ 255**	$C_{12}H_{20}O_2$ 253	$C_{12}H_{18}O_2$ 251	~~$C_{12}H_{16}O_2$ 249~~	~~$C_{12}H_{14}O_2$ 247~~	~~$C_{12}H_{12}O_2$ 245~~
13	$C_{13}H_{26}O_2$ 271	$C_{13}H_{24}O_2$ 269	$C_{13}H_{22}O_2$ 267	$C_{13}H_{20}O_2$ 265	~~$C_{13}H_{18}O_2$ 263~~	~~$C_{13}H_{16}O_2$ 261~~	~~$C_{13}H_{14}O_2$ 259~~
14	$C_{14}H_{28}O_2$ 285	**$C_{14}H_{26}O_2$ 283**	**$C_{14}H_{24}O_2$ 281**	$C_{14}H_{22}O_2$ 279	$C_{14}H_{20}O_2$ 277	~~$C_{14}H_{18}O_2$ 275~~	~~$C_{14}H_{16}O_2$ 273~~
15	$C_{15}H_{30}O_2$ 299	**$C_{15}H_{28}O_2$ 297**	$C_{15}H_{26}O_2$ 295	$C_{15}H_{24}O_2$ 293	$C_{15}H_{22}O_2$ 291	~~$C_{15}H_{20}O_2$ 289~~	~~$C_{15}H_{18}O_2$ 287~~
16	**$C_{16}H_{32}O_2$ 313**	$C_{16}H_{30}O_2$ 311	$C_{16}H_{28}O_2$ 309	$C_{16}H_{26}O_2$ 307	$C_{16}H_{24}O_2$ 305	$C_{16}H_{22}O_2$ 303	~~$C_{16}H_{20}O_2$ 301~~
17	$C_{17}H_{34}O_2$ 327	$C_{17}H_{32}O_2$ 325	**$C_{17}H_{30}O_2$ 323**	$C_{17}H_{28}O_2$ 321	$C_{17}H_{26}O_2$ 319	$C_{17}H_{24}O_2$ 317	~~**$C_{17}H_{22}O_2$ 315**~~
18	$C_{18}H_{36}O_2$ 341	$C_{18}H_{34}O_2$ 339	$C_{18}H_{32}O_2$ 337	$C_{18}H_{30}O_2$ 335	$C_{18}H_{28}O_2$ 333	$C_{18}H_{26}O_2$ 331	$C_{18}H_{24}O_2$ 329
19	**$C_{19}H_{38}O_2$ 355**	$C_{19}H_{36}O_2$ 353	$C_{19}H_{34}O_2$ 351	$C_{19}H_{32}O_2$ 349	$C_{19}H_{30}O_2$ 347	$C_{19}H_{28}O_2$ 345	$C_{19}H_{26}O_2$ 343
20	**$C_{20}H_{40}O_2$ 369**	$C_{20}H_{38}O_2$ 367	$C_{20}H_{36}O_2$ 365	$C_{20}H_{34}O_2$ 363	$C_{20}H_{32}O_2$ 361	$C_{20}H_{30}O_2$ 359	$C_{20}H_{28}O_2$ 357
21	$C_{21}H_{42}O_2$ 383	$C_{21}H_{40}O_2$ 381	$C_{21}H_{38}O_2$ 379	$C_{21}H_{36}O_2$ 377	$C_{21}H_{34}O_2$ 375	$C_{21}H_{32}O_2$ 373	**$C_{21}H_{30}O_2$ 371**
22	$C_{22}H_{44}O_2$ 397	$C_{22}H_{42}O_2$ 395	$C_{22}H_{40}O_2$ 393	$C_{22}H_{38}O_2$ 391	$C_{22}H_{36}O_2$ 389	$C_{22}H_{34}O_2$ 387	$C_{22}H_{32}O_2$ 385
23	**$C_{23}H_{46}O_2$ 411**	$C_{23}H_{44}O_2$ 409	$C_{23}H_{42}O_2$ 407	$C_{23}H_{40}O_2$ 405	$C_{23}H_{38}O_2$ 403	$C_{23}H_{36}O_2$ 401	$C_{23}H_{34}O_2$ 399
24	$C_{24}H_{48}O_2$ 425	$C_{24}H_{46}O_2$ 423	$C_{24}H_{44}O_2$ 421	**$C_{24}H_{42}O_2$ 419**	**$C_{24}H_{40}O_2$ 417**	$C_{24}H_{38}O_2$ 415	$C_{24}H_{36}O_2$ 413
25	$C_{25}H_{50}O_2$ 439	$C_{25}H_{48}O_2$ 437	$C_{25}H_{46}O_2$ 435	$C_{25}H_{44}O_2$ 433	$C_{25}H_{42}O_2$ 431	$C_{25}H_{40}O_2$ 429	$C_{25}H_{38}O_2$ 427
26	$C_{26}H_{52}O_2$ 453	$C_{26}H_{50}O_2$ 451	$C_{26}H_{48}O_2$ 449	$C_{26}H_{46}O_2$ 447	$C_{26}H_{44}O_2$ 445	$C_{26}H_{42}O_2$ 443	$C_{26}H_{40}O_2$ 441
27	$C_{27}H_{54}O_2$ 467	$C_{27}H_{52}O_2$ 465	$C_{27}H_{50}O_2$ 463	$C_{27}H_{48}O_2$ 461	**$C_{27}H_{46}O_2$ 459**	$C_{27}H_{44}O_2$ 457	$C_{27}H_{42}O_2$ 455
28	$C_{28}H_{56}O_2$ 481	$C_{28}H_{54}O_2$ 479	$C_{28}H_{52}O_2$ 477	$C_{28}H_{50}O_2$ 475	$C_{28}H_{48}O_2$ 473	$C_{28}H_{46}O_2$ 471	$C_{28}H_{44}O_2$ 469
29	$C_{29}H_{58}O_2$ 495	$C_{29}H_{56}O_2$ 493	$C_{29}H_{54}O_2$ 491	$C_{29}H_{52}O_2$ 489	$C_{29}H_{50}O_2$ 487	$C_{29}H_{48}O_2$ 485	$C_{29}H_{46}O_2$ 483
30	$C_{30}H_{60}O_2$ 509	$C_{30}H_{58}O_2$ 507	$C_{30}H_{56}O_2$ 505	$C_{30}H_{54}O_2$ 503	$C_{30}H_{52}O_2$ 501	$C_{30}H_{50}O_2$ 499	$C_{30}H_{48}O_2$ 497
31	$C_{31}H_{62}O_2$ 523	$C_{31}H_{60}O_2$ 521	$C_{31}H_{58}O_2$ 519	$C_{31}H_{56}O_2$ 517	$C_{31}H_{54}O_2$ 515	$C_{31}H_{52}O_2$ 513	$C_{31}H_{50}O_2$ 511
32	$C_{32}H_{64}O_2$ 537	$C_{32}H_{62}O_2$ 535	$C_{32}H_{60}O_2$ 533	$C_{32}H_{58}O_2$ 531	$C_{32}H_{56}O_2$ 529	$C_{32}H_{54}O_2$ 527	$C_{32}H_{52}O_2$ 525
33	$C_{33}H_{66}O_2$ 551	$C_{33}H_{64}O_2$ 549	$C_{33}H_{62}O_2$ 547	$C_{33}H_{60}O_2$ 545	$C_{33}H_{58}O_2$ 543	$C_{33}H_{56}O_2$ 541	$C_{33}H_{54}O_2$ 539

[a] Chemical formula corresponding to the expected mass

[b] Expected formula weight for a compound with the corresponding carbon number and Z family

[c] m/z values considered naphthenic acids after analyzing compounds in Figure 6.1 **(bold)**

Table 7.2 Summary of the mass and percentage of the major ion $[M+57]^+$, A+2 ion, and the product of a minor fragmentation event, the $[M+99]^+$ ion after GC-MS analyses of the *t*-butyldimethylsilyl derivatives of individual six pure compounds with molecular formula $C_nH_{2n+Z}O_2$.

Compound (C no., Z no.)	$[M+57]^+$ (m/z)	$[M+57]^+$ (%)[a]	A+2 ion (m/z) (C no., Z no.)[b]	A+2 ion (%)[a]	$[M+99]^+$ (m/z) (C no., Z no.)[b]	$[M+99]^+$ (%)[a]
1-Methyl-1-cyclo-hexanecarboxylic acid (8, -2)	199	86	201 (8, 0)	9	241 (11, -2)	3
trans-4-Pentylcyclo-hexanecarboxylic acid (12, -2)	255	84	257 (12, 0)	8	297 (15, -2)	2
Dicyclohexylacetic acid (14, -4)	281	89	283 (14, -2)	2	323 (17, -14)	0
2-Hexyldecanoic acid (16, 0)	313	72	315[c] (17, -12)[b]	10[c]	355 (19, 0)	3
Eicosanoic acid (20, 0)	369	59	371 (21, -12)	8	411 (23, 0)	2
5β-Cholanic acid (24, -8)	417	75	419 (24, -6)	8	459 (27, -8)	7

[a] Abundance of ion in %

[b] C no. and Z no. assigned from Table 7.1 based on m/z value.

[c] This m/z value does not conform to the chemical definition of a naphthenic acid given by Holowenko et al. (2002).

shown in Figure 7.2, a methyl group may also fragment, leading to $[M+99]^+$ fragments. This is a minor event, as established by St. John et al. (1998). In our experiments, they contribute between 0% (dicyclohexylacetic acid) and 7% (5β-cholanic acid) to the overall observed ions from a pure compound (Table 7.2). Stable isotopes resulting in the A+1 and A+2 ions have much greater influence on the ion intensity. The A+1 ion can constitute 8.7% (dicyclohexylacetic acid) to 40.5% (5β-cholanic acid) of the total signal (Table 7.3). The A+2 ion may constitute between 2% (5β-cholanic acid) to 10% (2-hexyldecanoic acid) of the total signal (Table 7.2). Highly abundant ions may contribute

Table 7.3 Summary of absolute and relative intensities of the A and A+1 ions from the GC-MS analyses of the individual six pure compounds with molecular formula $C_nH_{2n+Z}O_2$.

Compound (C no., Z no.)	A ion (m/z)[a]	Intensity of the A ion	A+1 ion (m/z)	Intensity of A+1 ion	Relative intensity of A+1 ion (%)[b]
1-Methyl-1-cyclohexane-carboxylic acid (8, -2)	199	182,000	200	35,000	19
trans-4-Pentyl-cyclohexanecarboxylic acid (12, -2)	255	405,000	256	70,000	17
Dicyclohexylacetic acid (14, -4)	281	1,048,000	282	91,000	9
2-Hexyldecanoic acid (16, 0)	313	471,000	314	118,000	25
Eicosanoic acid (20, 0)	369	233,000	370	68,000	29
5β-Cholanic acid (24, -8)	417	4,000	418	2,000	41

[a] These are the $[M+57]^+$ ions entered in Table 7.1 used to assign carbon number and Z family.
[b] 100 x (Intensity of A+1 ion)/(Intensity of A ion).

to the signal of the ion with m/z 2 Da higher. This is especially problematic because of the ^{30}Si atom (St. John et al. 1998).

The derivatization of the carboxylic group of a naphthenic acid yields a product that contains an atom of Si, in addition to the C, H, and O atoms from the naphthenic acid. The computer analyses of the average mass spectra obtained from the GC-MS method developed by Holowenko et al. (2002) do not account for all of the naturally occurring isotopes of C and Si. The most abundant isotopes of these two elements are ^{12}C (98.93%) and ^{28}Si (92.2297%) (International Union of Pure and Applied Chemistry 1991). Other isotopes of these elements and their natural abundances are ^{13}C (1.07%), ^{29}Si (4.6832%) and ^{30}Si (3.0872%) (International Union of Pure and Applied Chemistry 1991). I was not concerned about the other isotopes of H and O, because these elements are mainly consisted of a single isotope, with ^{1}H having an abundance of 99.9885%, and

^{16}O having an abundance of 99.757% (International Union of Pure and Applied Chemistry 1991).

The analysis of the GC-MS results, using the computer program described by Holowenko and Fedorak (2001), considers only the most abundant isotopes and uses the entries in Table 7.1 for the assignment of ions observed in the average mass spectrum to the appropriate carbon number and Z family. For example, consider an organic acid with the formula $C_{24}H_{40}O_2$ (Figure 7.1), having carbon number 24, and Z=-8. The *t*-butyldimethylsilyl derivative of a molecule of this compound has a molecular weight of 474, if only the most abundant isotopes are present in the product. The most abundant fragment of this compound is the $[M+57]^+$ ion (St. John et al. 1998), arising from $^{12}C_{24}{}^{1}H_{39}{}^{16}O_2{}^{28}Si(^{12}C^{1}H_3)_2^+$ and giving m/z=417. This value is bold in Table 7.1. Thus, the m/z=417 ions in the average mass spectrum are considered to originate from the naphthenic acids with the formula $C_{24}H_{40}O_2$. The m/z=417 ion can be referred to as the A peak (or ion) in which the main elemental formula is composed of only the most abundant isotopes (McLafferty 1980). To illustrate the effects of the other naturally occurring isotopes of C and Si, three examples will be considered.

First, if the group from the derivatizing agent contained ^{29}Si (which would occur in 4.6832% of the derivatized molecules) the resulting major fragment of the derivative would be $^{12}C_{24}{}^{1}H_{39}{}^{16}O_2{}^{29}Si(^{12}C^{1}H_3)_2^+$, which would give m/z=418. This is the so called A+1 peak (or ion) which is one mass unit greater than the A ion (McLafferty 1980). There is no entry in Table 7.1 with a value of 418, so this ion is ignored by the computer program. Thus, about 5% of the derivatized $^{12}C_{24}{}^{1}H_{40}{}^{16}O_2$ will not appear in the three-dimensional plot of the naphthenic acids distribution.

Second, if the organic acid contained one atom of ^{13}C (which would occur at a frequency of 1.07% per carbon atom in the molecule) the resulting major fragment of the derivative would be $^{13}C_1{}^{12}C_{23}{}^{1}H_{39}{}^{16}O_2{}^{28}Si(^{12}C^{1}H_3)_2^+$, which would give m/z=418. Again, there is no entry in Table 7.1 with a value of 418, and this ion is ignored by the computer program. In this example, there are 24 carbon atoms, so the predicted abundance of this A+1 ion is 24 x 1.07% or 26% Thus, about 26% of the derivatized organic acids with the nominal formula $C_{24}H_{40}O_2$ does not appear in the three-dimensional plot depicting naphthenic acids distribution. The amount of error introduced by ignoring the A+1 ion

from ^{13}C increases with the number of carbon atoms in the naphthenic acid. Hence, the relative abundance of the high molecular weight naphthenic acids may be underestimated by the GC-MS method.

Third, if the group from the derivatizing agent contained ^{30}Si (which would occur in 3.0872% of the derivatized molecules) the resulting major fragment of the derivative would be $^{12}C_{24}{}^{1}H_{39}{}^{16}O_2{}^{30}Si(^{12}C^{1}H_3)_2^+$, which would give m/z=419. This is the A+2 peak (or ion) which is two mass units greater than the A ion (McLafferty 1980). There is an entry in Table 7.1 with a value of 419, which is assigned as carbon number 24, Z=-6 (rather than -8) by the computer program. Thus, about 3% of the derivatized $^{12}C_{24}{}^{1}H_{40}{}^{16}O_2$ appears in the incorrect Z family in the three-dimensional plot of the naphthenic acids distribution.

There is also a small probability that the organic acid may contain two atoms of ^{13}C. The magnitude of this effect increases as the number of carbon atoms in the organic acid increase. This would produce a molecular ion that is two mass units higher than expected (i.e. an A+2 ion) if only ^{12}C atoms are considered. There could also be combinations of one atom of ^{13}C and one atom of ^{29}Si, again yielding an A+2 ion. All of these different combinations of isotopes would be expected to occur for each of the $[M+57]^+$ entries in Table 7.1.

Table 7.4 shows both non-corrected and corrected ion abundance for MLSB7, Syncrude B, Syncrude E, and Syncrude F. The last three samples were discussed in Chapters 3 and 4, MLSB7 was discussed in Chapter 3. The ions for each sample were divided into three groups: (1) C5 to C13, (2) C14 to C21, (3) C22+ cluster. The original (uncorrected) values were compared to the values corrected, as indicated in the materials and methods using an independent samples t-test after arcsine transformation, as discussed in Chapter 3. A $P<0.05$ means that there is a statistically significant difference between the two samples. I found that $P>0.05$ for all groups in all the samples, this meant that there was no statistically significant difference in the distribution of ions from the samples. Correcting for the theoretical effects of these isotopes does not significantly affect the observed three-dimensional plots (Table 7.4). Because of this, no correction for stable isotopes were performed on all other samples.

Table 7.4 Comparison of ion distributions of naphthenic acid ions not corrected (original), and corrected for stable isotope effects.

Naphthenic acids	Group	P[b]	Sums[a]	
			original	corrected
MLSB7	C5 to C13	0.639	20.7	18.8
	C14 to C21	0.928	60.2	58.6
	C22 to C33	0.525	19.0	22.6
Syncrude B	C5 to C13	0.601	15.9	14.7
	C14 to C21	0.988	78.5	78.8
	C22 to C33	0.454	5.6	6.5
Syncrude E	C5 to C13	0.710	22.0	20.4
	C14 to C21	0.976	71.2	71.7
	C22 to C33	0.541	6.9	7.9
Syncrude F	C5 to C13	0.540	26.7	24.4
	C14 to C21	0.961	61.1	61.6
	C22 to C33	0.615	12.2	14.0

[a] Total ion abundance in each specified group.
[b] Two samples are considered significantly different if $P<0.05$.

7.3.3 Determining the effect of structure on MS sensitivity

To determine if the sensitivity of the MS method is affected by the compound structure, the mixture of six compounds (Figure 7.1) was analyzed using GC-MS and plotted in three-dimensional graphs as shown in Figure 7.3. The six compounds have 9 to 24 carbons and 0 to 4 rings (Z=0 to -8). Figure 7.3A shows the ideal results from the GC-MS analysis of the mixture of six compounds. It shows that all the ions would have the same abundance if the reaction and ionization efficiencies were not affected by structure. Figure 7.3B shows the results observed from the analysis of a solution that contained equimolar concentrations of all six compounds. However, because the peaks corresponding to 2-hexyldecanoic acid, eicosanoic acid, and 5β-cholanic acid (Figure 7.3A peaks d, e, f) were not detected at 1% minimum occurrence, the concentration of these compounds were doubled in the mixture, resulting in Figures 7.3C and 7.3D. Figure 7.3D is the only one where 2-hexyldecanoic acid was detected in the graph. This was only after its signal exceeded the detection limit of the MS. In Chapter 5, the internal standard, *trans*-cinnamic acid was not used in subsequent experiments because it was not

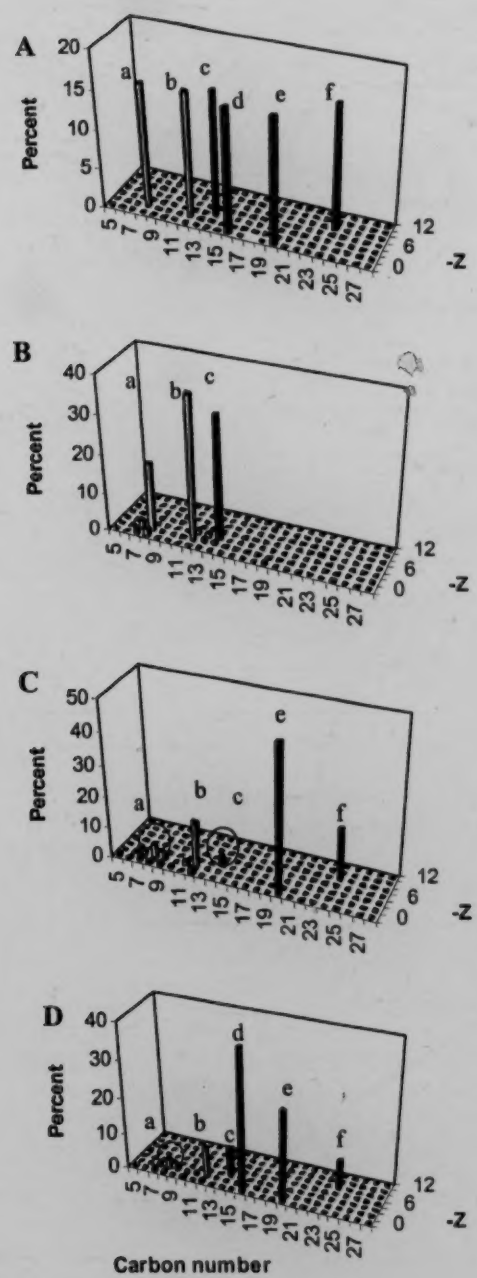


Figure 7.3 Three-dimensional plot GC-MS of *t*-butyldimethylsilyl derivatives of the mixture of six compounds: Theoretical plot of equimolar concentrations (A), observed plot with equimolar concentrations (B), twice the concentration for 2-hexyldecanoic acid, eicosanoic acid, and 5β-cholanic acid (C), and with the 2-hexyldecanoic acid signal too intense for the detector (D). Peaks correspond to methylcyclohexanecarboxylic acid (a), *trans*-4-pentylcyclohexanecarboxylic acid (b), dicyclohexylacetic acid (c), 2-hexyldecanoic acid (d), eicosanoic acid (e), and 5β-cholanic acid (f).

consistently included in the averaged spectrum at 1% minimum occurrence.

To help decide whether the observed differences in ion intensity were due to the MS or the derivatization procedure, the equimolar mixture of six derivatized surrogates was also analyzed by GC-AED. Figure 7.4 compares the standardized values for surrogate acid concentration, GC-AED, and GC-MS signals. Standardization to their corresponding percentage values was necessary because the three factors were measured using different units. Values for the six surrogates for each factor was summed, the value for a compound was then divided by the sum and multiplied by 100 to obtain the percentage. Since not all ions were detectable at 1% minimum occurrence (Figure 7.3B), data were also collected where the minimum occurrence variable was set at 0%. The latter allowed for the detection of all six ions. Ideally, the standardized percentage for concentration, GC-AED, and GC-MS should be equivalent if there was no discrimination between structures; this was not observed in Figure 7.4. Both GC-AED and GC-MS analyses were performed once, on the same sample.

Comparing the relative concentration and GC-AED signal shows some discrepancies; the GC-AED signal is lower than expected for methylcyclohexane-carboxylic acid and dicyclohexylacetic acid, and higher for eicosanoic acid. Aside from methylcyclohexanecarboxylic acid however, these discrepancies are minor. The derivatization reaction is therefore equally efficient, across the structure types tested (Figure 7.1). The decreased signal from methylcyclohexanecarboxylic acid may be due to volatilization, as a result of drying the sample under N_2 after derivatization. The more serious structure effects are seen with GC-MS results at both 0% and 1% minimum occurrence variable, which save for 2-hexyldecanoic acid, are much lower than expected. Although 2-hexyldecanoic acid gives the highest signal at 0% minimum occurrence, the GC peak is too narrow to show up at 1% minimum occurrence (Figure 7.3). That is, the derivatized 2-hexyldecanoic acid eluted as a very sharp peak from the GC, and the corresponding $m/z = 313$ ions were concentrated in a narrow region of the chromatogram, appearing in less than 1% of the scans.

Comparisons between ionization techniques from the literature have shown that the observed ion profile may change, depending on the method used. Fan (1991) showed

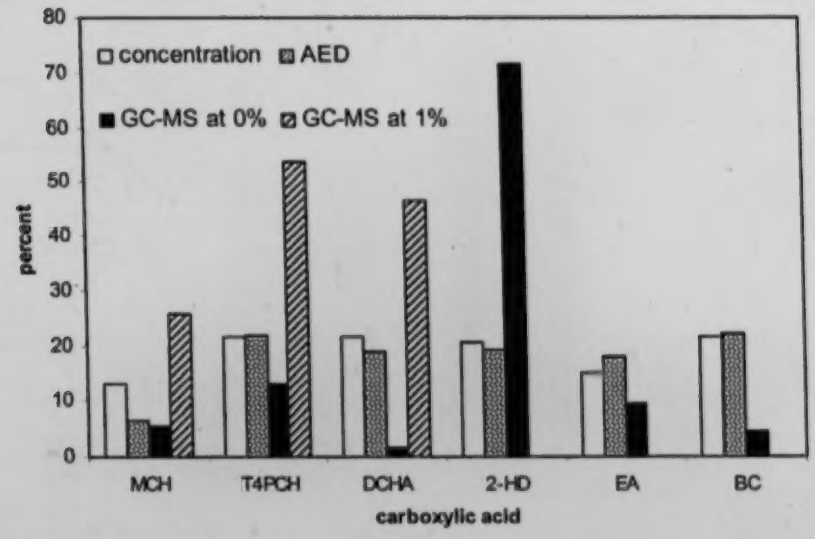


Figure 7.4 Comparison of normalized concentration, GC-AED signals, and GC-MS signals for a mixture of six acids containing methylcyclohexanecarboxylic acid (MCH), *trans*-4-pentylcyclohexanecarboxylic acid (T4PCH), dicyclohexylacetic acid (DCHA), 2-hexyldecanoic acid (2-HD), eicosanoic acid (EA), and 5β-cholanic acid (BC). GC-MS at 0% include all signals detected by the MS, GC-MS at 1% include ions which are included using the 1% minimum occurrence variable.

that fast atom bombardment mass spectrometry detects a wider range of formula weights compared to the chemical ionization method performed by Dzidic et al. (1988). Hsu et al. (2000) in turn determined that these higher formula weight compounds result from cluster ions. Hsu et al. (2000) compared four ionization methods, and determined that results from all four were slightly different. Analysis of the mixture of six compounds (Figure 7.1), as summarized in Figure 7.4 has shown that not all compounds were detected with equal efficiency by the GC-MS method used in this thesis. This means that the three-dimensional profiles discussed here, as well as in Holowenko et al. (2002) may not represent the true abundance of the different ions which constitute a naphthenic acids mixture.

7.3.4 Conclusion

From these analyses, it can be concluded that the stable isotopes have an

insignificant influence on the three-dimensional plots obtained after GC-MS. After isotope corrections, the abundance of higher molecular weight compounds increase slightly, but this difference is statistically insignificant. The more important factors, which affect the ions observed, are differences in detection sensitivity due to differences in molecular structure.

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8. OVERALL DISCUSSION AND SUGGESTIONS FOR FUTURE RESEARCH

8.1 Discussion

The main focus of this project was to characterize the ion distribution of naphthenic acids from oil sands ores and to evaluate the biodegradation of naphthenic acid mixtures by acclimated microbial communities, enriched from tailings water. To do this, several methods were developed, including statistical analyses, and an HPLC method utilizing NPH derivatives of naphthenic acids.

So far, TAN (ASTM D664, ASTM D974 Drews 1998) is the most common method of describing naphthenic acids concentration (Brient et al. 1995, Drews 1998, Slavcheva et al. 1999). TAN measures the acidity of the sample, and is therefore not specific to naphthenic acids. FT-IR analysis after extracting the sample into CH_2Cl_2 has also been widely applied (Jivraj et al. 1995, Holowenko et al. 2001, Rogers et al. 2002). Other methods used to measure concentrations include IR of the carbonyl group fluoroester (Yu and Green 1989), GC-FID (Herman et al. 1994, Jones et al. 2001), and MS-electrospray ionization (Headley et al. 2002, Rogers et al. 2002). FT-IR, fluoroester IR, and GC-FID have low sensitivity, and require samples dissolved in organic solvent. Large sample volumes are therefore needed for these analyses.

As described in Chapter 6, monitoring the biodegradation of naphthenic acids require analyzing several samples over the incubation time. Using the methods described above would require a large amount of space, as a consequence of monitoring large sample volumes. The development of an HPLC method described in Chapter 2 allowed the analysis of only 50 to 100 μL of samples. This meant that the biodegradation experiments could be done in 125 mL serum bottles. It allowed for a more efficient use of space. It was also faster and more accessible than currently available methods. All methods of analyzing naphthenic acids concentrations make use of the carboxylic acid moiety. This group was also used as a means of detection by HPLC. As described in Section 1.5.2, the molar extinction coefficient of carboxylic acid groups is low. As in

fatty acids, it was necessary to form naphthenic acids derivatives to promote detection. Among the methods available, derivatization with NPH (Miwa et al. 1985; Miwa and Yamamoto 1986, 1987; Miwa 2000) was the most convenient. As described in Chapter 2, this does not require extraction, and is amenable to UV-visible detection. Gradient elution, and taking the sum of eluted humps allowed concentrations to be determined for most of the compounds which make up a naphthenic acids mixture. Chapter 2 also shows that standard curves can be generated from Kodak acid, Merichem acid, and MLSBF, which means that samples containing them can be analyzed using this method. A decrease in naphthenic acids concentration can be monitored using HPLC.

Naphthenic acids composition is also an important consideration. Holowenko et al. (2002) found that tailings ponds which contained low naphthenic acids concentrations are less toxic, and have different distributions compared to those with higher concentrations. Different sources of petroleum may have different naphthenic acids distributions. Barrow et al. (2003) observed the same ions from petroleum obtained from two different sites, the distribution of these ions however, differed. Chapters 3 and 4 have been the first attempts to perform statistical analyses on distribution of these ions. Statistical analysis of the three-dimensional graphs generated per Holowenko et al. (2002) was attempted as described in Chapters 3 and 4. Chapter 3 was done initially. The method described allowed the comparison of two samples using an independent samples t-test after arcsine transformation. Logit transformation, and simultaneous comparisons of 15 ore samples were performed afterward, as described in Chapter 4. Logit transformation was an improvement over arcsine transform in terms of improving normality and variance. The statistical comparison itself was similar to a t-test. Comparisons between the arcsine and pairwise comparison results showed that the two methods may give different conclusions. In addition, hierarchical cluster analysis of the logit transformed data was also performed. The agreement between cluster analysis and pairwise comparisons depended on the cluster solution chosen. It is therefore evident that the kind of data transformation and data analysis performed affects the conclusions. The decision as to which method is better suited to analyzing GC-MS analyses of naphthenic acids largely depend on other variables such as site characteristics, which can help delineate which samples should be grouped together.

It was also found that the GC-MS method itself can affect the observed naphthenic acids composition. St. John et al. (1998) believed that ions which are very abundant could affect the observed abundance of ions with one less unsaturation unit. The effects of stable isotopes of carbon and silicon were therefore discussed in Chapter 7. High resolution MS, as well as GC coupled to low resolution MS, both showed that minor fragmentation events and stable isotopes affect the observed abundance of the base peak. The A+1 ion has the greatest influence, which increases with increasing carbon number. Comparisons between uncorrected and corrected three-dimensional graphs, via t-test of arcsine transformed data, showed that the ion distributions were not statistically different ($P>0.05$). Chapter 7 shows that stable isotopes do not greatly influence the observed ions. St. John et al. (1998) showed that active site substitution in phenolic compounds affects their reactivity towards MTBSTFA. Yu and Green (1989) determined the effect of structure on fluoroester formation. In Chapter 7, a test was done to determine how much effect structure has on the observed three-dimensional graphs. Chapter 7 shows that different compounds were not detected with equal efficiency by the GC-MS. Comparing the molar concentration of the individual compounds with signals obtained using GC-AED proved that all compounds were derivatized equally, differences in volatility was also not a factor. The mass spectrometer is therefore responsible for the observed discrepancies.

Besides evaluating methods to detect and characterize naphthenic acids, their biodegradation was also determined. This is shown in Chapter 6, with the biodegradation of Kodak salts, and Merichem refined acids. Previous works have postulated possible aerobic biodegradation pathways for a surrogate naphthenic acid, cyclohexane carboxylic acid (Blakley 1974, Kaneda 1974, Rho and Evans 1975, Smith and Callely 1975, Blakley 1978, Taylor and Trudgill 1978, Blakley and Papish 1982, Herman et al. 1994, Dutta and Harayama 2001). There are two main pathways which have been established: one is ring cleavage followed by β-oxidation, whereas the other goes through aromatization prior to ring fission.

There have also been various studies on the biodegradation of commercial and tailings pond naphthenic acids (Herman et al. 1994, Lai et al. 1996, Holowenko et al. 2002). It has been previously established that a decrease in naphthenic acids

concentration is associated with a decrease in toxicity (MacKinnon and Boerger 1986, Herman et al. 1994). The association between concentration, toxicity, and compound distribution as determined by Holowenko et al. (2002) is, however, less understood. The differences in three-dimensional graphs, and the increase in the proportion of the C22+ cluster in particular is believed to be due to the selective biodegradation of some components of the naphthenic acids mixture over the others.

Chapter 6 is an attempt to find an explanation for the observations by Holowenko et al. (2002) under controlled laboratory conditions. As described by Herman et al. (1994), there was a decrease in toxicity, an increase in carbon dioxide production, increase in colony forming units, and a decrease in naphthenic acids concentrations; (determined by HPLC) for both Merichem refined, and Kodak salts naphthenic acids. Methods established in Chapters 2 and 5 were used to analyze the biodegradation samples. HPLC as described in Chapter 2 was used to determine changes in naphthenic acids concentrations, extractions for GC-MS analysis were done as established in Chapter 5.

The differences in naphthenic acids composition between Kodak salts and Merichem acids as established in Chapters 3 and 4 may explain the differences in the rates of biodegradation, as well as the differences in the change in compound distribution observed in the two commercial naphthenic acids preparation after biodegradation. Higher molecular weight compounds are evident in the three-dimensional graphs of day 7 Merichem acids, as well as day 18 Kodak salts. Those in the Merichem acids were not visible previously, in the case of Kodak salts; an increase in the C22+ cluster was observed.

There are two possible explanations for these compounds. High molecular weight compounds not previously observable because of low concentrations have become enriched because of selective biodegradation of other compounds, as postulated by Holowenko et al. (2002). As St. John et al. (1998) has shown, alcohol moieties may be derivatized; a second explanation then is that a naphthenic acid metabolite may be derivatized twice through either two carboxylic acid groups or a combination of a carboxylic acid and an alcohol group. The biodegradation of Merichem acids in Chapter 6 has shown that Z families of 0 and -2 are preferentially biodegraded. The

biodegradation of group 1 over group 2 (as described in Chapter 3) is also exhibited for both Merichem acids and Kodak salts. However, because neither mixture contained significant or even detectable amounts of group 3 compounds initially, the biodegradation rate of this group cannot be compared to groups 1 and 2. Nevertheless, it can be concluded that some components are preferentially biodegraded over others.

Both Chapters 2 and 6 show the biodegradation of refined Merichem naphthenic acids. The rate of biodegradation differed. There was a 40% decrease in Merichem acids concentration in Chapter 2, whereas there was a 93% decrease in Chapter 6. The mixed cultures used for both sets of experiments were acclimated from the same tailings pond water sample. However, they were different sets of acclimated culture. Whereas those used in Chapter 2 were eventually transferred every week prior to using them in the experiment, those used in Chapter 6 were transferred every month. They were also different sub-samples from the same tailings water sample. This means that the discrepancy in the amount of Merichem acid biodegraded is due to differences in the characteristics or identity of the microbial community. Moore et al. (2002) for example, found that different microbial communities exist in naphthenic acid contaminated sites, compared to non-contaminated sites using BIOLOG™. A study on fatty acid ester biodegradation by a microbial community (Sonderkamp et al. 2001) found that the major component of the population may not be responsible for the biodegradation. The same may be true for the community associated with naphthenic acids biodegradation. The minor community members may not always be included in the inoculum used for acclimation on the naphthenic acids.

8.2 Suggestions for Future Research

Both the biodegradation of naphthenic acids and the methods used in their analysis need to be further characterized. The biodegradation of extracts from tailings ponds needs to be characterized, as was done for Kodak salts and Merichem acids. Herman et al. (1994) showed that these extracts can be biodegraded by microbial communities from the tailings ponds, though only 20% could be mineralized, compared to about 50% of the Kodak salts. This is important, since one of the main concerns

expressed at the beginning of the project was the biodegradability of tailings pond extracts.

Although addressed in the Introduction, the question of which fractions of the naphthenic acids mixtures are more toxic was not fully answered. This was because the decrease in toxicity was associated with both a change in the ion fingerprint, as well as a decrease in naphthenic acid concentration. A means of answering this may be to fractionate a mixture of naphthenic acids. This requires much work, as described by Seifert and Teeter (1969). An alternative would be to extract naphthenic acids at different points along a biodegradation experiment. In the experiments performed here, and other works (Herman et al. 1994, Holowenko et al. 2002), the concentration is a factor in the toxicity test. A decrease in toxicity may therefore be due to a decrease in naphthenic acids concentration, not necessarily because the most toxic components were biodegraded. If the concentration can be held constant while measuring toxicity during a biodegradation experiment, then it could be determined whether the most toxic components were in fact biodegraded. Since Chapter 2 has established that HPLC is a quick, reliable method of determining naphthenic acids concentrations in laboratory cultures, the concentrations may be appropriately adjusted given the results from this analysis. According to the biodegradation of Kodak salts and Merichem acids in Chapter 6, this requires the concentration of volumes 4 to 20 times those extracted in this project. A second HPLC analysis is then required to account for the recovery error involved in extraction. The extraction procedure performed in Chapter 2 may be used, provided that acetic acid does not interfere with the Microtox assay. Otherwise an alternative extraction method with the same efficiency is required.

In Chapter 6 the source of compounds with higher molecular weights, not previously observed, could not be conclusively attributed to the presence of compounds with two reactive sites. Although the prospect of having concentrated these compounds through selective biodegradation of the rest of the envelope is unlikely, it is still possible. Another method for MS analysis, which does not require derivatization, is therefore necessary to settle the question. The high molecular weight compounds would not be visible if they were a result of derivatizing two reactive groups in the same compound. In addition, if the proportions of these ions increase with incubation time, using the alternate

method, then it can be concluded that higher molecular weight compounds are more resistant to biodegradation.

The limitations of the MS method as a means of comparing different naphthenic acids mixtures have been addressed in this study. Not all ions were included in the average at 1% minimum occurrence. A standard method of analyzing naphthenic acids mixtures should therefore be developed if it becomes necessary to compare different samples, as was done in Chapter 4.

The method of comparison must also be standardized. By comparing Chapters 3 and 4, it was evident that the method used to compare the data affects the outcome. The difficulty in analyzing naphthenic acids is that common statistical tools formally assume that data sets have equal variance and normal distribution (Ramsey and Schafer 1997). This is not true when the data are viewed across carbon or –Z number. Commonly used statistical tools are comparisons between means and the data for each sample are assumed to be distributed around a common value: the greater the sample size, the closer the measured value approaches the mean. This is generally not the case with naphthenic acids. The naphthenic acids analyzed in this study have distributions consisting of peaks and valleys. Unlike data which do approach a similar value – the mean, the mean of the GC-MS data along each group described in Chapters 3 and 4 represent the averaged abundance. It is therefore difficult for the data to have equal variance, and finding similarities where there are differences, and differences where there is none occurs. Additional information is needed to meaningfully interpret the differences and similarities between ore samples in Chapter 4.

As Chapter 2 and 6 resulted in different measured amounts of Merichem acids biodegraded, it would be of interest to learn more about the abundance and characteristics of the microorganisms responsible. If the differences were due to different rates of transferring the culture, then the community must change through the course of the experiment. The reasons for this should also be elucidated. The lower biodegradation in Chapter 2 may be because only part of the community necessary for Merichem biodegradation was acclimated; or genes responsible for producing key enzymes may fail to transfer after each generation because there was no advantage in doing so. The two explanations differ in that for the first, the community itself changes, whereas in the

second, the abilities of individuals in the community changed. Differences in the characteristics of the population may affect how they deal with toxicity, biodegradation of more resistant compounds in the mixture, and catabolism of intermediates.

8.3 Literature Cited

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A. APPENDIX

A.A Effects of transformation on data structure of four naphthenic acids preparations

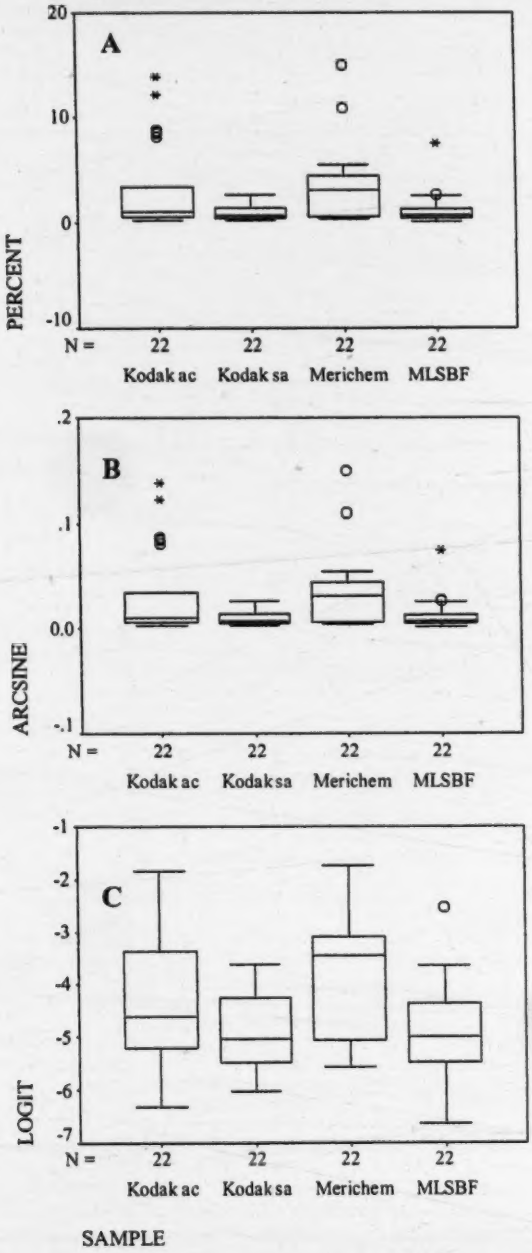

Figure A1 Group 1 non-transformed (A), arcsine transformed (B), and logit transformed (C) GC-MS data for four naphthenic acids: Kodak acids (Kodak ac), Kodak salts (Kodak sa), Refined Merichem (Merichem), and a 7-year old tailings pond water (MLSB7). Outliers are represented as open circles, and extreme outliers are shown as asterisks.

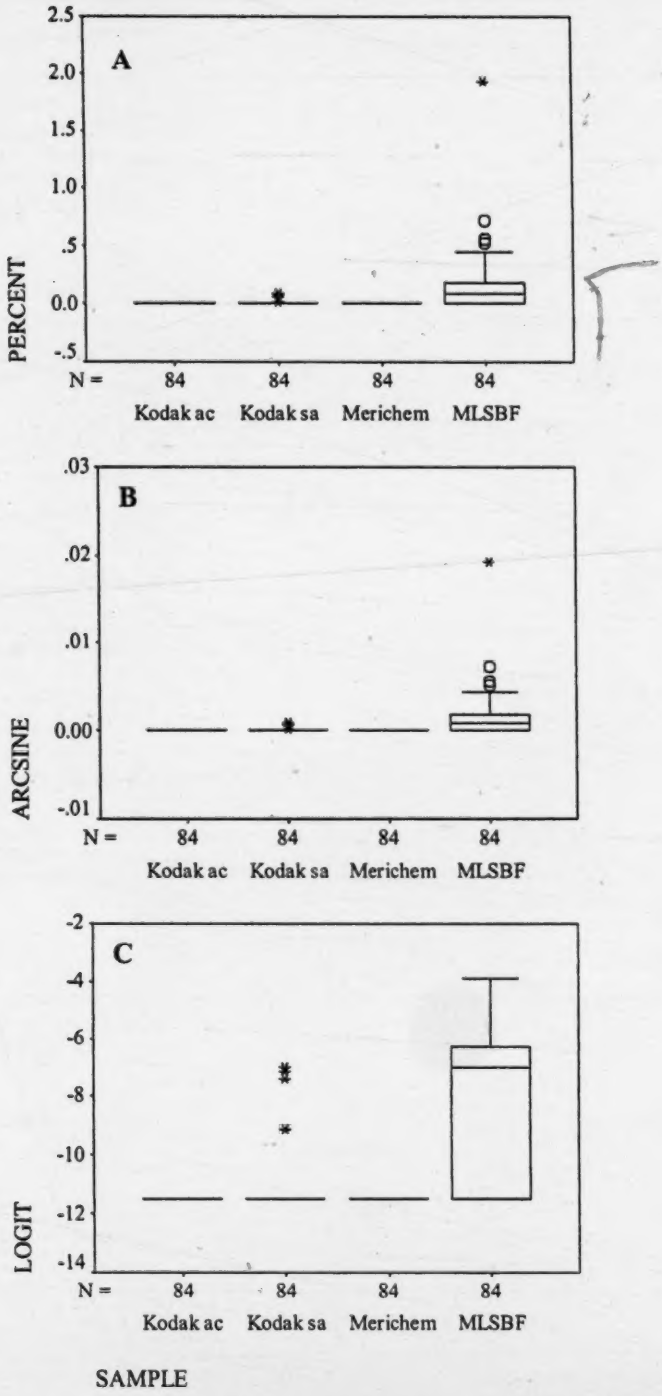

Figure A2 Group 3 non-transformed (A), arcsine transformed (B), and logit transformed (C) GC-MS data for four naphthenic acids: Kodak acids (Kodak ac), Kodak salts (Kodak sa), Refined Merichem (Merichem), and a 7-year old tailings pond water (MLSB7). Outliers are represented as open circles, and extreme outliers are shown as asterisks.

A.B Three-dimensional graphs of oil sands ore samples

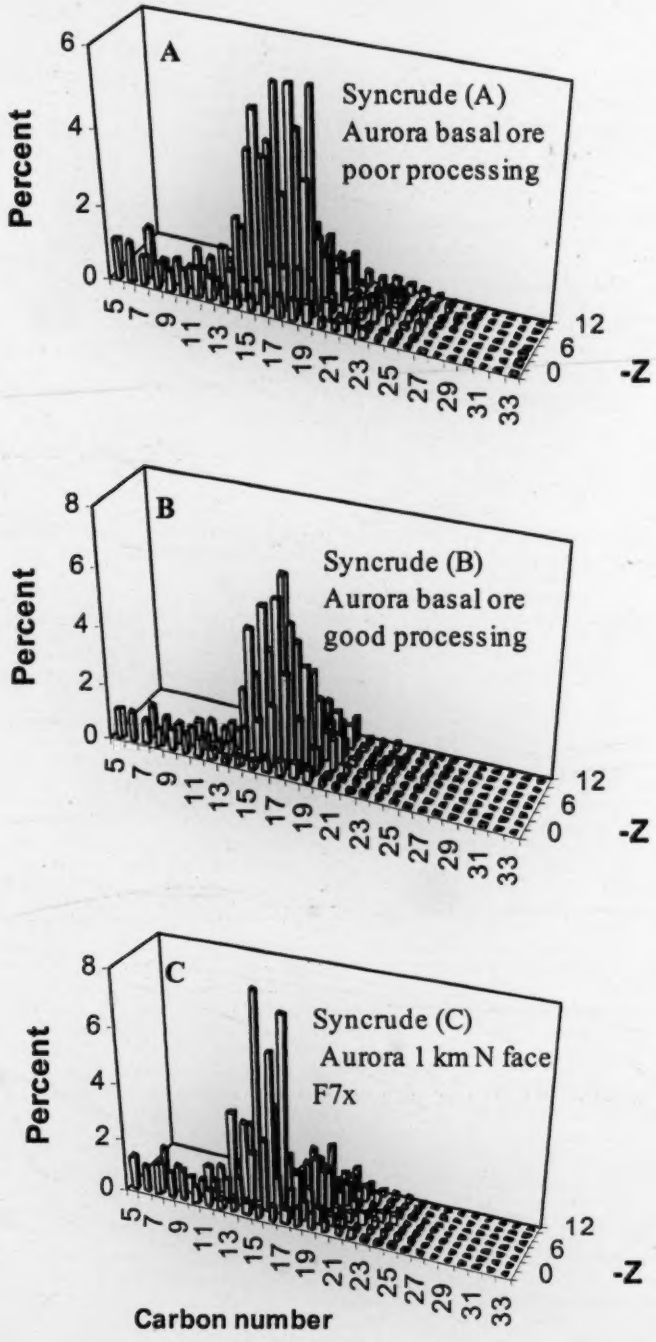

Figure A3 Three-dimensional plots after GC-MS analysis of oil sands ores from Syncrude A (A), B (B), and C (C).

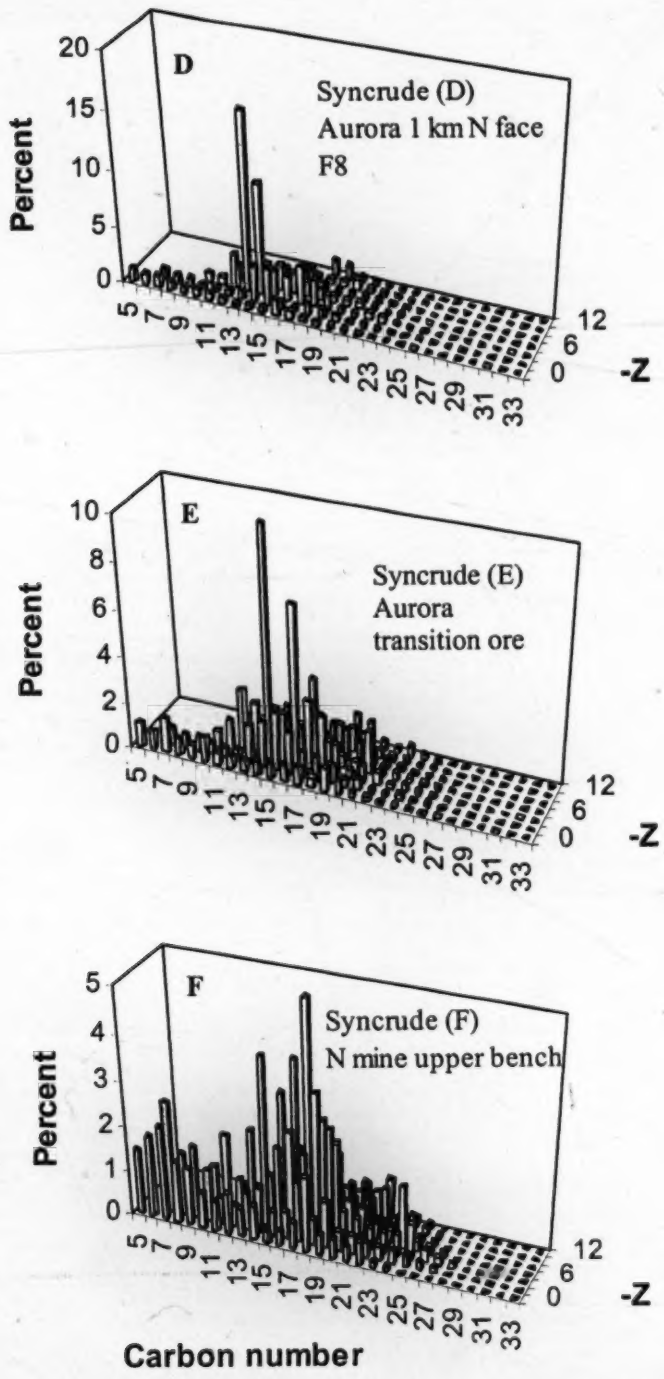

Figure A4 Three-dimensional plots after GC-MS analysis of oil sands ores from Syncrude D (D), E (E), and F (F).

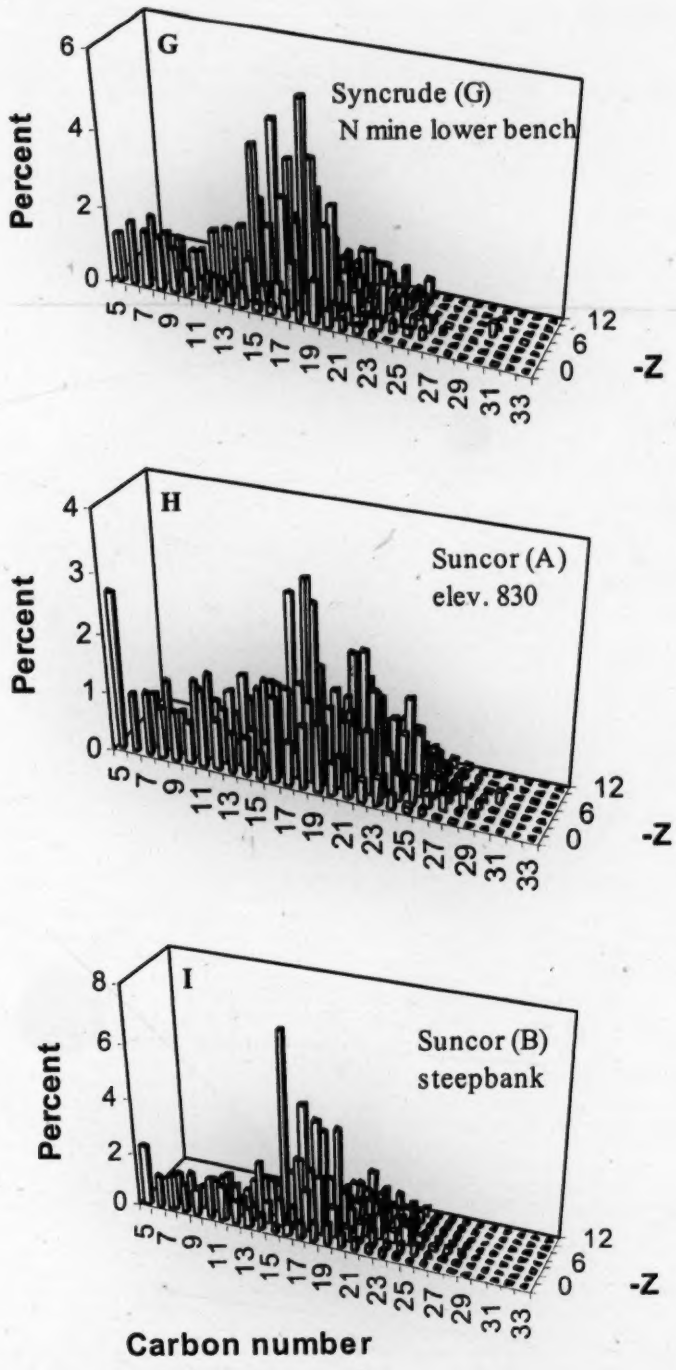

Figure A5 Three-dimensional plots after GC-MS analysis of oil sands ores from Syncrude G (G), Suncor A (H), and B (I).

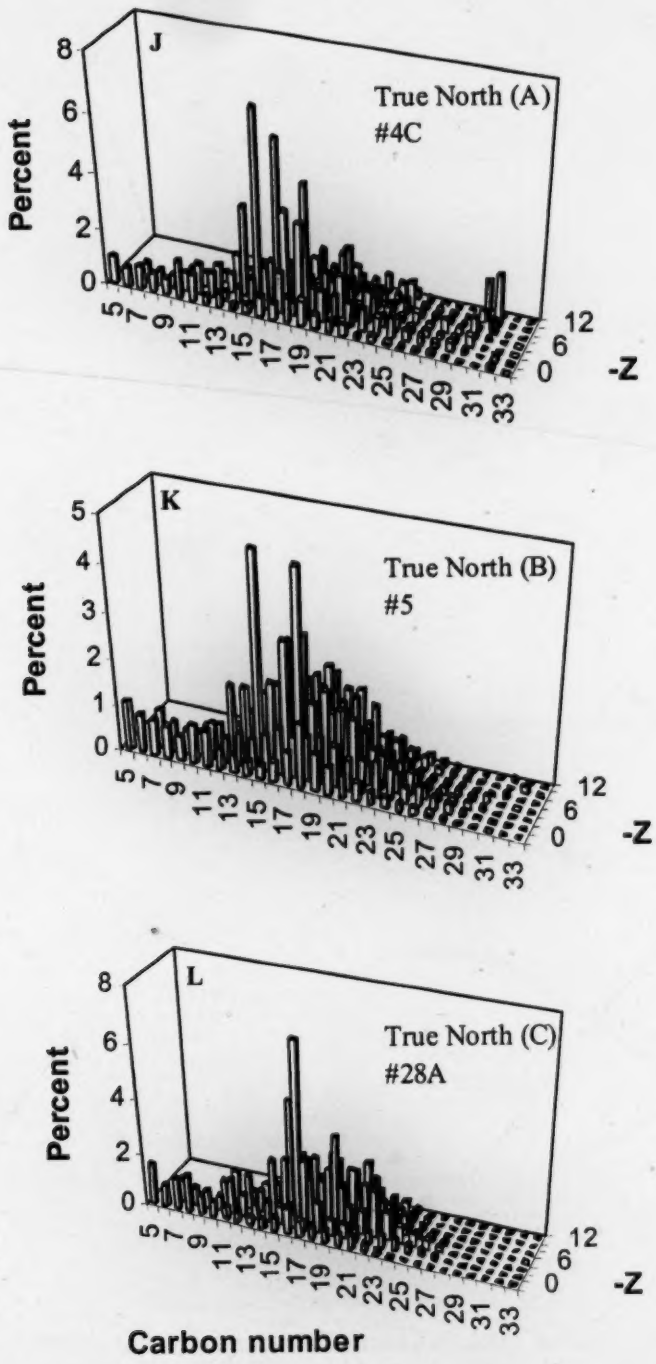

Figure A6 Three-dimensional plots after GC-MS analysis of oil sands ores from True North A (J), B (K), C (L).

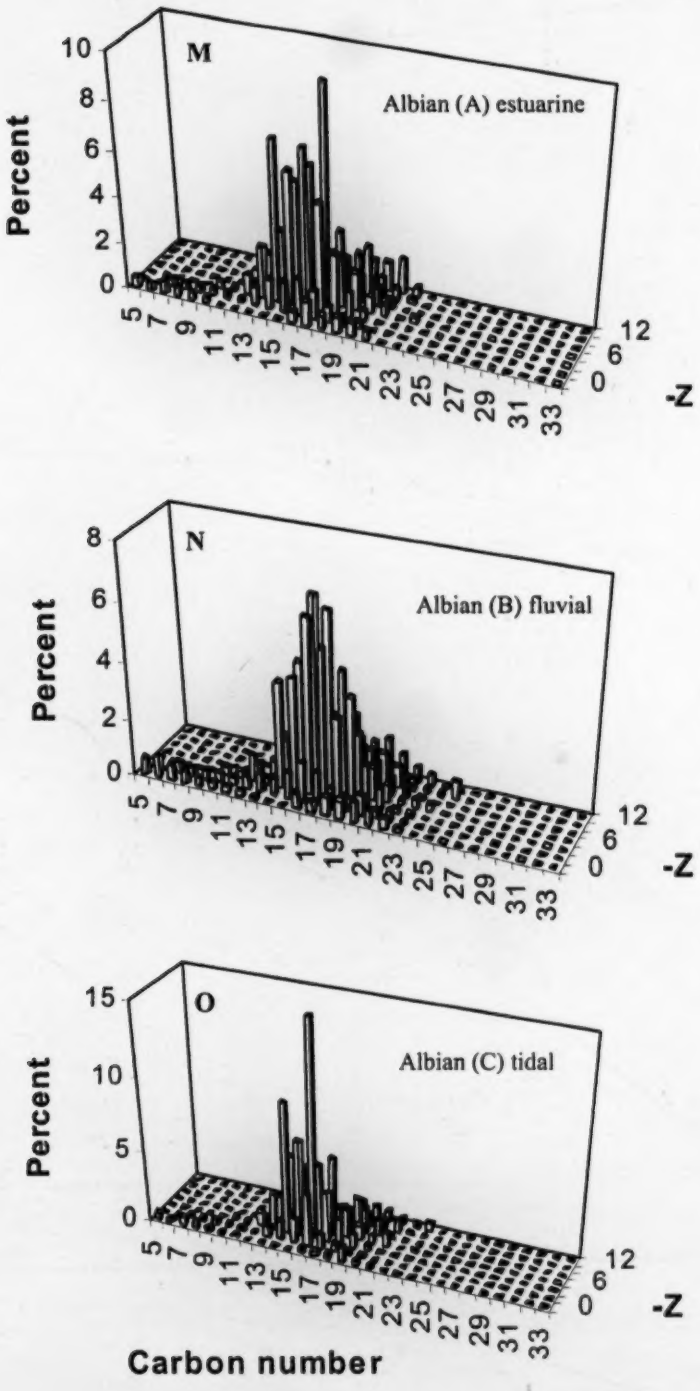

Figure A7 Three-dimensional plots after GC-MS analysis of oil sands ores from Albian A (M), B (N), C (O).

A.C Normalized data (in percent) after GC-MS analysis of 15 Athabasca oil sands ore samples

Syncrude (A) Aurora basal ore poor processing

C number	Z family 0	-2	-4	-6	-8	-10	-12	% by C no.
5	1.09							1.09
6	1.07							1.07
7	0.78	1.39						2.17
8	0.66	0.57						1.23
9	0.57	0.69						1.26
10	0.68	0.55	0.85					2.08
11	0.81	0.60	0.68					2.09
12	0.65	0.38	1.08	0.51				2.62
13	0.47	0.73	1.95	1.52				4.67
14	0.31	0.53	3.78	4.63	0.63			9.89
15	0.37	0.61	3.66	3.86	0.82			9.32
16	0.56	1.12	5.53	2.60	1.15	0.55		11.52
17	0.60	1.14	5.57	4.32	1.15	0.99		13.77
18	0.57	1.18	3.30	5.44	1.78	0.95	0.88	14.09
19	0.57	0.97	1.91	1.22	1.58	0.76	0.69	7.70
20	0.22	0.51	0.50	0.87	0.52	0.81	0.79	4.22
21	0.24	0.19	0.41	0.27	0.21	0.22	0.37	1.91
22	0.35	0.35	0.22	0.36	0.21	0.18	0.30	1.97
23	0.26	0.18	0.44	0.36	0.43	0.30	0.37	2.33
24	0.07	0.07	0.26	0.32	0.58	0.18	0.28	1.76
25	0.07	0.07	0.14	0.22	0.08	0.07	0.22	0.86
26	0.06	0.06	0.29	0.37	0.20	0.13	0.16	1.28
27	0.00	0.00	0.07	0.07	0.07	0.19	0.06	0.46
28	0.00	0.00	0.00	0.08	0.18	0.00	0.00	0.26
29	0.00	0.00	0.00	0.00	0.07	0.09	0.04	0.19
30	0.00	0.00	0.00	0.00	0.00	0.00	0.04	0.04
31	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
32	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
33	0.00	0.05	0.11	0.00	0.00	0.00	0.00	0.16
% by Z no.	11.04	11.93	30.73	27.03	9.66	5.42	4.19	100.00

Syncrude (B) Aurora basal ore good processing

C number	Z family 0	-2	-4	-6	-8	-10	-12	% by C no.
5	1.05							1.05
6	1.01							1.01
7	0.84	1.11						1.95
8	0.66	0.77						1.43
9	0.72	0.67						1.39
10	0.70	0.65	0.62					1.97
11	0.63	0.50	0.74					1.88
12	0.37	0.39	0.59	0.56				1.91
13	0.19	0.55	0.68	1.87				3.28
14	0.32	1.11	4.31	2.52	0.52			8.78
15	0.32	1.47	5.18	3.47	0.56			11.00
16	0.48	2.11	5.53	6.12	2.50	0.51		17.24
17	0.51	3.32	4.77	3.93	1.83	1.05		15.42
18	0.58	1.86	3.42	3.02	1.28	0.96	1.20	12.32
19	0.49	0.96	2.40	1.00	1.09	1.19	0.59	7.71
20	0.23	0.16	1.10	1.01	0.53	0.84	0.82	4.69
21	0.14	0.18	0.25	0.26	0.20	0.16	0.18	1.37
22	0.10	0.20	0.19	0.18	0.14	0.11	0.16	1.08
23	0.14	0.11	0.42	0.34	0.23	0.13	0.24	1.61
24	0.10	0.09	0.21	0.19	0.31	0.14	0.14	1.17
25	0.05	0.05	0.08	0.12	0.06	0.08	0.08	0.51
26	0.03	0.03	0.09	0.18	0.11	0.12	0.06	0.62
27	0.01	0.01	0.01	0.08	0.08	0.07	0.05	0.32
28	0.01	0.01	0.00	0.05	0.05	0.01	0.01	0.14
29	0.00	0.00	0.00	0.01	0.03	0.03	0.01	0.10
30	0.00	0.03	0.00	0.00	0.00	0.00	0.02	0.05
31	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
32	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
33	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
% by Z no.	9.68	16.34	30.58	24.91	9.52	5.41	3.56	100.00

Figure A8 Syncrude A and B data matrix summarizing percent for each ion.

Syncrude (C) Aurora 1 km N face F7x

C number	Z family 0	-2	-4	-6	-8	-10	-12	% by C no.
5	1.26							1.26
6	0.96							0.96
7	1.20	1.52						2.72
8	0.98	0.93						1.91
9	1.16	0.66						1.82
10	0.65	0.66	0.89					2.19
11	0.61	0.72	1.00					2.32
12	0.34	0.90	3.15	0.99				5.38
13	0.41	1.06	2.88	2.60				6.96
14	0.46	2.11	7.65	1.93	0.62			12.78
15	0.41	2.67	5.61	3.48	0.64			12.82
16	0.55	1.42	7.01	1.77	0.83	0.58		12.17
17	0.55	1.06	1.37	1.30	1.29	1.55		7.11
18	0.73	0.69	0.97	1.83	1.61	0.96	1.53	8.32
19	0.63	0.59	1.75	1.42	0.63	0.61	0.62	6.27
20	0.45	0.42	0.46	0.77	0.86	0.85	0.90	4.70
21	0.33	0.43	0.38	0.55	0.72	0.58	0.42	3.40
22	0.36	0.21	0.47	0.23	0.24	0.42	0.28	2.21
23	0.11	0.27	0.32	0.34	0.17	0.18	0.23	1.61
24	0.13	0.25	0.21	0.31	0.25	0.12	0.21	1.46
25	0.11	0.18	0.14	0.09	0.11	0.13	0.07	0.83
26	0.06	0.14	0.09	0.12	0.03	0.03	0.03	0.49
27	0.04	0.05	0.03	0.07	0.02	0.02	0.02	0.27
28	0.00	0.00	0.02	0.00	0.00	0.00	0.00	0.02
29	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
30	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
31	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
32	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
33	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
% by Z no.	12.49	16.94	34.41	17.80	8.02	6.03	4.31	100.00

Syncrude (D) Aurora 1 km N face F8

C number	Z family 0	-2	-4	-6	-8	-10	-12	% by C no.
5	1.19							1.19
6	0.93							0.93
7	0.99	0.95						1.95
8	0.82	0.71						1.53
9	0.68	0.69						1.37
10	0.71	0.50	0.76					1.97
11	0.63	0.41	0.68					1.72
12	0.44	0.50	3.01	1.23				5.18
13	0.28	0.65	15.70	1.50				18.13
14	0.47	3.12	9.75	1.99	0.46			15.80
15	0.45	3.16	2.41	2.18	1.35			9.55
16	1.45	0.63	2.06	1.77	0.83	1.08		7.83
17	0.77	0.52	3.25	1.20	1.70	0.53		7.97
18	0.45	0.99	2.42	0.84	0.67	0.49	1.37	7.22
19	0.40	0.78	1.25	0.58	0.52	0.54	1.20	5.27
20	0.33	0.52	0.44	0.46	0.43	0.91	0.53	3.62
21	0.24	0.24	0.36	0.58	0.58	0.38	0.38	2.77
22	0.17	0.39	0.65	0.44	0.44	0.24	0.26	2.59
23	0.09	0.15	0.56	0.18	0.31	0.17	0.21	1.67
24	0.08	0.14	0.20	0.37	0.12	0.11	0.10	1.12
25	0.00	0.10	0.13	0.09	0.00	0.13	0.00	0.44
26	0.00	0.09	0.09	0.00	0.00	0.00	0.00	0.18
27	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
28	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
29	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
30	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
31	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
32	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
33	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
% by Z no.	11.58	15.25	43.71	13.41	7.41	4.59	4.05	100.00

Figure A9 Syncrude C and D data matrix summarizing percent for each ion.

Syncrude (E) Aurora Transition ore

C number	Z family 0	-2	-4	-6	-8	-10	-12	% by C no.
5	1.05							1.05
6	0.84							0.84
7	1.54	0.81						2.35
8	0.76	0.63						1.39
9	0.63	0.65						1.28
10	1.10	0.51	0.72					2.33
11	0.52	0.42	1.33					2.26
12	0.53	0.50	2.81	1.12				4.97
13	0.46	1.72	2.34	0.98				5.51
14	0.60	1.98	9.98	1.89	1.17			15.63
15	0.57	2.52	2.16	2.13	0.67			8.05
16	0.68	1.76	6.96	1.49	0.80	0.53		12.21
17	0.75	1.07	2.94	3.60	1.71	0.53		10.59
18	0.46	0.96	2.41	1.01	1.48	1.08	0.56	7.97
19	0.97	0.77	1.33	0.48	1.34	1.08	1.35	7.32
20	0.63	0.34	0.38	0.38	0.88	0.95	1.13	4.69
21	0.28	0.46	0.67	1.83	0.70	0.37	0.39	4.70
22	0.20	0.20	0.52	0.41	0.42	0.26	0.28	2.28
23	0.11	0.14	0.17	0.32	0.30	0.17	0.47	1.68
24	0.08	0.24	0.31	0.21	0.10	0.11	0.23	1.27
25	0.08	0.09	0.20	0.16	0.07	0.15	0.13	0.87
26	0.07	0.15	0.07	0.11	0.05	0.05	0.05	0.54
27	0.06	0.05	0.05	0.06	0.00	0.00	0.00	0.22
28	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
29	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
30	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
31	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
32	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
33	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
% by Z no.	12.97	15.97	35.34	16.18	9.70	5.26	4.58	100.00

Syncrude (F) N mine upper bench

C number	Z family 0	-2	-4	-6	-8	-10	-12	% by C no.
5	1.47							1.47
6	1.82							1.82
7	2.10	2.48						4.58
8	1.36	1.40						2.76
9	1.27	1.63						2.89
10	0.79	1.15	1.09					3.03
11	0.64	0.59	1.84					3.08
12	0.90	0.47	0.91	0.59				2.87
13	0.64	0.83	2.09	0.63				4.20
14	0.77	1.00	3.73	0.98	0.90			7.38
15	0.27	0.53	1.80	2.87	0.47			5.94
16	0.33	0.60	2.23	3.65	1.56	0.42		8.79
17	0.59	1.33	1.79	4.96	0.78	1.12		10.56
18	1.67	0.57	3.17	2.45	0.84	1.63	1.23	11.56
19	0.46	1.03	2.48	2.03	0.75	0.69	0.63	8.07
20	0.44	0.44	0.73	0.78	0.67	0.67	0.73	4.47
21	0.35	0.41	0.62	0.70	1.09	0.52	0.59	4.29
22	0.50	0.33	0.49	0.40	0.34	0.87	0.93	3.85
23	0.16	0.30	0.47	0.34	0.27	0.29	0.36	2.18
24	0.09	0.31	0.49	1.39	0.21	0.41	0.18	3.07
25	0.00	0.27	0.79	0.23	0.15	0.18	0.11	1.74
26	0.00	0.19	0.27	0.18	0.12	0.09	0.00	0.85
27	0.00	0.13	0.18	0.13	0.00	0.00	0.00	0.44
28	0.00	0.00	0.11	0.00	0.00	0.00	0.00	0.11
29	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
30	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
31	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
32	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
33	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
% by Z no.	16.62	16.02	25.28	22.30	8.15	6.88	4.76	100.00

Figure A10 Syncrude E and F data matrix summarizing percent for each ion.

Syncrude (G) N mine lower bench

C number	Z family 0	-2	-4	-6	-8	-10	-12	% by C no.
5	1.30							1.30
6	1.67							1.67
7	1.50	1.71						3.21
8	1.41	1.33						2.74
9	1.19	1.27						2.46
10	0.68	1.01	0.85					2.54
11	0.54	0.48	1.44					2.46
12	0.68	0.42	1.57	0.51				3.17
13	0.55	0.67	1.72	0.53				3.47
14	0.53	1.10	3.90	2.33	0.73			8.60
15	0.21	0.51	1.88	4.45	0.43			7.48
16	0.24	0.65	2.71	3.52	1.87	0.56		9.55
17	0.67	1.27	2.16	5.11	0.87	1.21		11.30
18	0.45	0.57	3.84	2.90	1.68	1.10	0.98	11.52
19	1.16	0.47	2.19	2.53	0.90	0.75	0.71	8.70
20	0.46	0.41	0.74	0.86	0.73	0.72	0.85	4.78
21	0.32	0.37	0.63	0.83	0.40	1.10	0.58	4.23
22	0.24	0.31	0.47	0.37	0.30	0.81	0.38	2.89
23	0.14	0.30	1.01	0.32	0.26	0.29	0.61	2.94
24	0.00	0.28	0.40	0.57	0.29	0.23	0.14	1.91
25	0.00	0.22	0.27	0.16	0.45	0.66	0.00	1.76
26	0.00	0.27	0.39	0.14	0.00	0.00	0.00	0.80
27	0.00	0.00	0.14	0.00	0.00	0.00	0.00	0.14
28	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
29	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
30	0.00	0.00	0.00	0.38	0.00	0.00	0.00	0.38
31	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
32	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
33	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
% by Z no.	13.95	13.64	26.32	25.50	8.92	7.43	4.24	100.00

Suncor (A) elev. 830

C number	Z family 0	-2	-4	-6	-8	-10	-12	% by C no.
5	2.67							2.67
6	1.00							1.00
7	1.07	0.93						2.00
8	0.85	1.19						2.04
9	0.85	0.72						1.56
10	0.72	1.31	0.79					2.82
11	1.31	1.45	0.89					3.65
12	0.84	0.92	1.11	0.58				3.45
13	0.68	0.41	1.43	0.89				3.41
14	0.66	0.67	1.24	1.25	0.65			4.48
15	0.45	0.49	1.41	1.24	0.48			4.07
16	1.45	0.39	1.34	2.82	0.64	0.71		7.34
17	0.74	0.46	1.48	3.08	0.92	0.40		7.08
18	1.08	1.36	2.85	1.70	0.85	0.47	0.55	8.86
19	1.15	1.00	1.18	1.35	0.85	0.99	1.61	8.13
20	0.56	0.54	1.00	1.05	0.78	1.47	1.16	6.57
21	0.48	1.14	0.88	2.09	1.52	1.14	0.95	8.20
22	0.39	1.08	1.61	1.11	0.87	0.54	0.60	6.20
23	0.49	0.37	0.62	1.08	0.69	0.83	0.48	4.54
24	0.26	0.47	1.01	1.47	0.71	0.70	0.30	4.93
25	0.12	0.46	1.06	0.53	0.45	0.43	0.21	3.26
26	0.13	0.21	0.37	0.29	0.28	0.16	0.14	1.59
27	0.03	0.30	0.27	0.20	0.18	0.11	0.09	1.17
28	0.00	0.06	0.26	0.06	0.04	0.04	0.03	0.49
29	0.00	0.08	0.11	0.04	0.03	0.00	0.00	0.25
30	0.00	0.00	0.03	0.23	0.00	0.00	0.00	0.26
31	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
32	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
33	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
% by Z no.	17.97	16.00	20.94	21.07	9.93	7.99	6.11	100.00

Figure A11 Syncrude G and Suncor A data matrix summarizing percent for each ion.

Suncor (B) steepbank

C number	Z family 0	-2	-4	-6	-8	-10	-12	% by C no.
5	2.23							2.23
6	1.17							1.17
7	1.25	1.10						2.35
8	1.04	1.20						2.25
9	0.97	0.85						1.82
10	1.49	1.17	0.99					3.65
11	1.30	1.48	0.79					3.58
12	0.83	0.74	0.77	0.84				3.18
13	0.65	0.46	1.89	1.09				4.09
14	0.50	0.74	1.34	0.92	0.67			4.18
15	0.26	0.95	6.85	1.49	0.72			10.27
16	0.44	0.78	2.33	4.12	0.60	0.63		8.90
17	0.56	1.52	2.02	3.62	1.20	0.42		9.34
18	0.66	2.12	3.58	1.54	1.01	0.47	0.46	9.85
19	0.75	1.10	3.75	1.14	0.68	0.96	0.70	9.07
20	0.47	0.46	0.87	0.88	0.75	0.82	1.25	5.51
21	0.41	0.76	0.68	1.08	0.73	0.79	0.70	5.15
22	0.30	0.58	0.90	0.52	0.50	0.38	0.59	3.77
23	0.23	0.27	0.40	0.63	0.35	0.43	0.33	2.63
24	0.21	0.31	0.67	0.69	0.46	0.42	0.28	3.04
25	0.07	0.39	0.46	0.26	0.26	0.24	0.12	1.80
26	0.04	0.13	0.21	0.16	0.22	0.14	0.08	0.97
27	0.02	0.09	0.23	0.10	0.10	0.10	0.05	0.69
28	0.00	0.06	0.14	0.09	0.04	0.02	0.02	0.38
29	0.00	0.02	0.05	0.02	0.00	0.00	0.00	0.10
30	0.00	0.00	0.00	0.03	0.00	0.00	0.00	0.03
31	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
32	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
33	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
% by Z no.	15.88	17.29	28.93	19.22	8.30	5.81	4.56	100.00

True North (A) #4C

C number	Z family 0	-2	-4	-6	-8	-10	-12	% by C no.
5	0.99							0.99
6	0.69							0.69
7	0.84	0.69						1.53
8	0.67	0.53						1.20
9	0.59	0.50						1.08
10	1.35	0.69	0.56					2.60
11	0.95	1.07	0.54					2.57
12	0.32	0.86	0.89	0.37				2.44
13	0.52	0.33	0.67	1.12				2.64
14	0.23	0.54	3.16	0.67	0.28			4.88
15	0.35	0.97	6.59	1.04	0.59			9.54
16	0.70	1.52	1.44	5.43	0.44	0.25		9.78
17	0.60	1.44	3.28	1.24	0.51	0.32		7.40
18	0.89	0.48	2.99	4.06	0.56	0.97	0.40	10.36
19	0.92	0.43	0.81	0.74	1.63	0.49	0.50	5.52
20	0.53	1.10	1.48	0.89	0.48	1.34	1.28	7.11
21	0.45	1.05	1.46	0.46	0.82	0.99	0.42	5.65
22	0.40	0.90	0.42	0.31	0.65	0.34	0.36	3.37
23	0.16	0.79	1.12	0.81	0.62	0.34	0.64	4.48
24	0.25	0.63	0.41	0.44	0.31	0.30	0.43	2.77
25	0.11	0.71	0.40	0.24	0.52	0.84	0.28	3.11
26	0.09	0.38	0.26	0.45	0.14	0.13	0.09	1.55
27	0.07	0.30	0.21	0.17	0.20	0.09	0.07	1.11
28	0.17	0.19	0.18	0.46	0.08	0.06	0.05	1.18
29	0.06	0.32	0.19	0.26	0.07	0.04	0.00	0.93
30	0.00	0.14	0.38	0.73	0.06	0.41	1.15	2.86
31	0.00	0.00	0.00	0.07	0.49	1.77	0.00	2.34
32	0.14	0.18	0.00	0.00	0.00	0.00	0.00	0.31
33	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
% by Z no.	13.04	16.74	27.45	19.97	8.45	8.68	5.67	100.00

Figure A12 Suncor B and True North A data matrix summarizing percent for each ion.

True North (B) #5

C number	Z family 0	-2	-4	-6	-8	-10	-12	% by C no.
5	1.08							1.08
6	0.85							0.85
7	0.78	0.86						1.64
8	0.71	0.64						1.35
9	0.57	0.53						1.10
10	0.86	0.57	0.51					1.94
11	0.81	0.82	0.63					2.26
12	0.46	0.55	1.54	0.52				3.08
13	0.39	0.72	1.56	0.88				3.55
14	0.34	0.68	4.51	1.36	0.44			7.32
15	0.31	0.84	1.79	1.60	0.82			5.35
16	0.45	1.03	2.73	2.59	1.37	0.26		8.43
17	0.74	0.92	4.32	2.76	1.69	0.64		11.07
18	1.42	0.99	1.54	1.98	1.57	1.88	1.30	10.68
19	0.84	1.04	1.86	1.25	1.95	1.16	1.27	9.38
20	0.63	0.91	1.53	1.32	0.98	1.31	1.33	8.00
21	0.51	0.68	1.38	0.93	0.82	0.81	1.02	6.16
22	0.42	0.75	0.49	0.63	0.49	0.59	0.49	3.86
23	0.28	0.79	0.73	0.66	0.46	0.55	0.42	3.89
24	0.22	0.69	0.41	0.49	0.34	0.41	0.23	2.79
25	0.18	0.33	0.51	0.32	0.20	0.32	0.20	2.06
26	0.15	0.44	0.29	0.19	0.11	0.14	0.13	1.46
27	0.11	0.29	0.19	0.14	0.07	0.10	0.08	0.96
28	0.07	0.22	0.17	0.14	0.08	0.05	0.01	0.74
29	0.01	0.09	0.14	0.09	0.01	0.02	0.00	0.35
30	0.00	0.04	0.08	0.16	0.00	0.00	0.00	0.28
31	0.00	0.00	0.00	0.00	0.11	0.00	0.09	0.20
32	0.00	0.00	0.00	0.00	0.00	0.15	0.00	0.15
33	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
% by Z no.	13.18	15.42	26.92	18.02	11.51	8.39	6.57	100.00

True North (C) #28A

C number	Z family 0	-2	-4	-6	-8	-10	-12	% by C no.
5	1.55							1.55
6	0.85							0.85
7	1.19	0.89						2.08
8	1.40	0.66						2.06
9	0.74	0.65						1.39
10	0.61	0.55	0.89					2.05
11	0.51	1.31	1.37					3.19
12	0.40	0.83	1.29	0.43				2.95
13	0.30	0.55	0.79	0.82				2.46
14	0.36	0.74	2.09	0.91	0.28			4.37
15	0.48	0.70	2.26	4.21	0.36			8.01
16	0.98	0.83	6.62	2.26	0.63	0.74		12.06
17	0.60	2.65	2.42	2.26	1.30	0.45		9.69
18	0.46	1.14	1.73	1.91	2.26	1.71	0.54	9.76
19	0.49	0.58	3.49	1.31	0.93	1.45	1.14	9.39
20	0.45	0.55	0.95	2.08	0.77	0.75	1.58	7.14
21	0.51	0.52	0.90	1.62	1.27	1.35	0.61	6.79
22	0.28	0.47	1.77	0.51	0.84	0.44	0.45	4.78
23	0.16	0.46	1.03	0.84	0.33	0.34	0.39	3.56
24	0.05	0.43	0.64	0.75	0.53	0.36	0.22	2.98
25	0.00	0.34	0.44	0.28	0.18	0.19	0.13	1.55
26	0.00	0.25	0.33	0.22	0.07	0.00	0.00	0.88
27	0.00	0.16	0.21	0.10	0.00	0.00	0.00	0.46
28	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
29	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
30	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
31	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
32	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
33	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
% by Z no.	12.39	15.25	29.22	20.52	9.77	7.78	5.07	100.00

Figure A13 True North B and True North C data matrix summarizing percent for each ion.

Albian (A) estuarine

C number	Z family 0	-2	-4	-6	-8	-10	-12	% by C no.
5	0.49							0.49
6	0.30							0.30
7	0.57	0.32						0.89
8	0.47	0.29						0.76
9	0.33	0.32						0.65
10	0.25	0.30	0.29					0.84
11	0.00	0.32	0.20					0.51
12	0.00	0.16	0.90	0.24				1.30
13	0.00	0.83	2.26	1.65				4.73
14	0.00	1.25	6.97	2.81	0.61			11.64
15	0.00	1.32	5.76	5.04	0.94			13.07
16	0.51	1.58	6.83	5.83	1.69	0.58		17.02
17	1.07	1.22	4.66	9.33	1.91	0.93		19.13
18	0.62	0.70	2.61	3.26	1.80	0.90	1.35	11.26
19	0.71	0.38	1.09	1.60	1.17	2.11	1.20	8.25
20	0.62	0.30	0.42	0.73	0.78	0.90	1.23	4.99
21	0.41	0.00	0.00	0.50	0.36	0.53	1.51	3.31
22	0.00	0.00	0.00	0.00	0.00	0.21	0.41	0.62
23	0.00	0.00	0.00	0.24	0.00	0.00	0.00	0.24
24	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
25	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
26	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
27	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
28	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
29	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
30	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
31	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
32	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
33	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
% by Z no.	6.34	9.29	31.99	31.24	9.27	6.17	5.70	100.00

Albian (B) fluvial

C number	Z family 0	-2	-4	-6	-8	-10	-12	% by C no.
5	0.58							0.58
6	0.76							0.76
7	0.55	0.40						0.95
8	0.43	0.30						0.73
9	0.33	0.32						0.64
10	0.31	0.32	0.28					0.90
11	0.25	0.31	0.23					0.78
12	0.17	0.46	0.93	0.37				1.93
13	0.09	0.65	0.24	0.53				1.51
14	0.00	0.61	3.71	1.60	0.41			6.33
15	0.00	0.90	3.95	4.27	0.97			10.10
16	0.55	1.37	6.05	6.56	1.97	0.37		16.88
17	0.40	1.13	5.13	6.14	2.40	0.74		15.94
18	0.66	0.70	2.87	4.25	2.13	2.26	1.17	14.04
19	0.65	0.48	3.69	2.20	1.48	1.12	1.16	10.77
20	0.73	0.69	1.45	1.13	1.06	1.11	1.38	7.55
21	0.54	0.28	0.74	0.64	0.59	0.73	0.92	4.42
22	0.39	0.38	0.16	0.19	0.48	0.42	0.56	2.57
23	0.17	0.12	0.09	0.34	0.13	0.20	0.39	1.43
24	0.00	0.00	0.00	0.27	0.00	0.11	0.13	0.52
25	0.00	0.00	0.00	0.00	0.00	0.65	0.00	0.65
26	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
27	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
28	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
29	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
30	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
31	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
32	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
33	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
% by Z no.	7.53	9.41	29.53	28.49	11.62	7.71	5.71	100.00

Figure A14 Albian A and B data matrix summarizing percent for each ion.

Albian (C) tidal

C number	Z family							
	0	-2	-4	-6	-8	-10	-12	% by C no.
5	0.45							0.45
6	0.29							0.29
7	0.56	0.31						0.87
8	0.45	0.27						0.72
9	0.28	0.34						0.61
10	0.20	0.23	0.21					0.65
11	0.12	0.18	0.15					0.45
12	0.00	0.12	0.94	0.12				1.18
13	0.00	0.75	2.51	1.82				5.08
14	0.00	1.25	9.08	4.85	0.29			15.47
15	0.00	1.66	6.57	4.35	0.74			13.32
16	0.34	3.59	15.34	4.67	1.29	0.58		25.80
17	0.33	0.97	3.39	5.30	1.41	0.90		12.29
18	0.44	0.54	1.71	1.99	1.19	1.71	1.03	8.60
19	1.05	0.27	0.89	0.96	1.75	0.81	0.87	6.60
20	0.35	0.15	0.33	0.52	1.13	0.60	0.79	3.87
21	0.22	0.00	0.00	0.93	0.27	0.76	0.47	2.65
22	0.00	0.00	0.00	0.00	0.00	0.17	0.26	0.43
23	0.00	0.00	0.00	0.15	0.00	0.00	0.50	0.65
24	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
25	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
26	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
27	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
28	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
29	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
30	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
31	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
32	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
33	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
% by Z no.	5.08	10.62	41.13	25.66	8.04	5.54	3.93	100.00

Figure A15 Albian C data matrix summarizing percent for each ion.